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SECTION MEETINGS

CLEVELAND	
University Hospitals	October 9, 1952
ILLINOIS	
Baxter Laboratories	October 13, 1952
IOWA	
State University of Iowa	October 28, 1952
PACIFIC COAST	
University of California	October 15, 1952
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A Convenient Method for Determining Chlorides in Urine: Modification of Northrop's Potentiometric Titration.* (19868)

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(Introduced by E. M. Landis.)

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To meet a need for rapidly determining the chloride concentration in a large number of small specimens of urine and other biological fluids the method of potentiometric titration recently described by Northrop(1) was selected because of its simplicity and precision. The great convenience of this method was confirmed; but the values obtained with urine were found to be in error because of interference by urates. Modified solutions eliminating such interference are being described because 4 years' experience has shown that the method as modified (a) is much faster than other methods in common use once the equipment has been assembled, (b) is quite suitable for small samples, and (c) provides a much more satisfactory end-point, facilitating greater precision even in the hands of a beginner.

Apparatus and methods. The method, based on the well-established principles of potentiometric titration(2), consists of the titration of the chloride with a standard solution of silver nitrate in the presence of an appropriate electrolyte, using the voltage of a silver electrode to indicate the end-point.

The diagram of the apparatus shown in Fig. 1, modified from Northrop(1), specifies the composition of the solutions in terms of their final molarity, the number of significant

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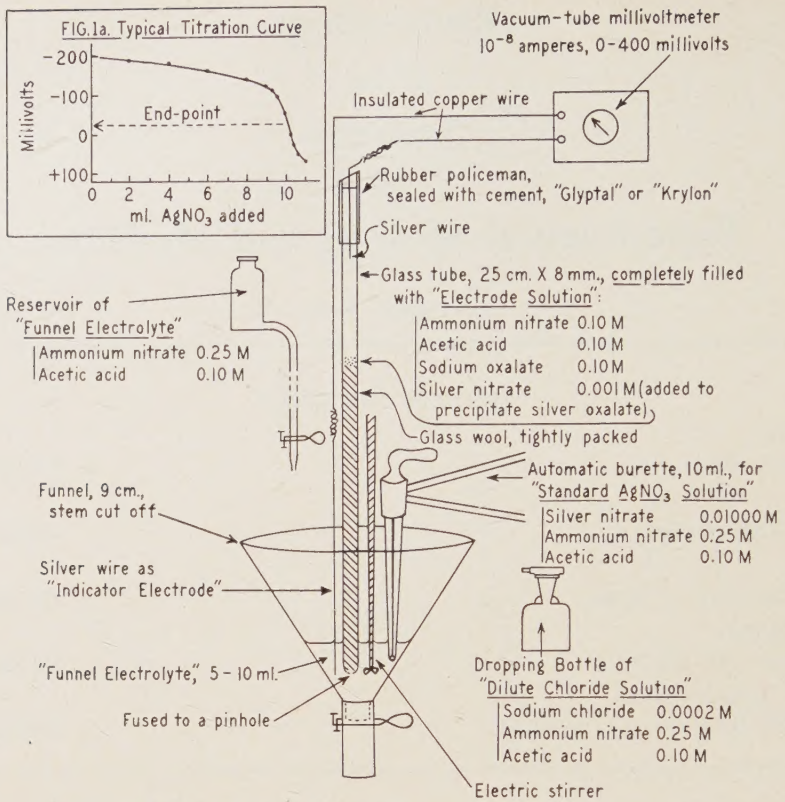

FIG. 1. Arrangement of apparatus and composition of solutions.
FIG. 1a (Inset). Typical titration curve, from which meter reading at end-point is determined.

figures indicating the requisite accuracy. The essential modification is the use of ammonium nitrate and acetic acid to maintain an acid pH while keeping liquid junction potentials insignificant. The "Standard $AgNO_3$ Solution" is accurately prepared from pure dried $AgNO_3$. It is stable for months when protected from light. The "Dilute Chloride Solution" is conveniently prepared by adding a drop of isotonic saline to about 50 ml of "Funnel Electrolyte." The reference or oxalate half-cell, formed by the silver wire in the tube of "Electrode Solution," is stable indefinitely if its lower tip is not allowed to dry. We have had to refill this tube with "Electrode Solution" twice in 4 years, because of leaks. A titration curve is performed when a new oxalate half-cell is prepared: plotting voltmeter readings in arbitrary units against ml $AgNO_3$ added (Fig. 1a). Theoretically, the steepest point of this curve is the equivalence point. (Actually, the chloride or indicator electrode is then about 20 to 50 millivolts negative to the oxalate electrode.) As this point is quite constant for any given oxalate half-cell, this meter reading (without calibration to convert into millivolts) is used as the end-point throughout the life of the half-cell (years). The titration curve can be repeated if doubt ever arises as to the condition of either electrode.

Since, at the end-point, the voltage between the electrodes is not too far from zero, and is changing at the rate of 7 to 40 millivolts per micro-equivalent (0.1 ml) of $AgNO_3$ added (depending mainly upon the amount of fluid and protein in the funnel), and since the same meter reading is used at the beginning and end of each titration, analytical results are very insensitive to large percentage errors in the actual voltage selected as end-point. An accurate potentiometric instrument is therefore quite unnecessary. A very simple, conventional, vacuum-tube millivoltmeter of a continuously indicating type was substituted

TABLE I. Comparison of Determinations by Potentiometric Titration with Values Obtained by Other Methods.

Nature of unknown	Spec. No.	Ref.*	Chloride conc. of specimen (mEq/L) Mean ± stand. dev. (No. of titrations) Ref. method	Potentiometric	Diff.	Aliquot size (ml)
Pure NaCl	A	I	50	49.7 ± .1 (2)	— .3	.5
	A	I	50	50 ± .4 (4)	0	1
	B	I	100	99.8 ± .6 (3)	— .2	.2
	C	I	100	99.8 ± .5 (5)	— .2	.5
	D	I	160	159.5 ± .3 (3)	— .5	.5
	D	I	160	160.2 ± .1 (4)	+ .2	1
	E	I	200	201.3 ± .8 (3)	+1.3	.2
	F	I	200	198.9 ± .3 (4)	—1.1	.5
Rat urine	A	IV	74 ± .2 (4)	73.3 ± .0 (5)	— .7	.5
	B	IV	99 ± .6 (6)	98.7 ± .2 (5)	— .3	.5
	C	IV	139.2 ± .5 (7)	139.5 ± .1 (5)	+ .3	.5
Human urine	A	IV	60.8 ± .3 (5)	60.4 ± .1 (5)	— .4	.5
	B	IV	108.6 ± .4 (5)	108 ± .1 (5)	— .6	.5
	C	IV	140.7 ± .3 (5)	140.3 ± .2 (5)	— .4	.5
80-85% evaporated milk sol. with NaCl added	A	IIIa	110.3 ±2 (3)		+4.8	
	A	IIIb	112.4 ± .3 (4)	115.1 ± .9 (4)	+2.7	.5
	A	II	115.5 ± .9 (3)		— .4	
	B	IIIa	111.4 ±1.4 (3)		+2.8	
	B	II	112.9 ± .6 (4)	114.2 ± .7 (4)	+1.3	.5
	B	IIIb	113.4 ± .4 (3)		+ .8	
	C	IV	104.2 ± .1 (5)		+2.5	
	C	V	108.1 ±1.2 (6)	106.7 ± .3 (5)	—1.4	.5
Beef serum	A	II	98.7 ± .2 (4)		+2.4	
	A	IV	98.9 ± .6 (6)	101.1 ± .4(17)	+2.2	.5, 1
	A	VI		99 ± .3 (5)	+2.1	1
	A	VII		98.5 ± .6(10)	+2.6	1

* Reference method used:

I Accurate preparation.
II Open Carius method of Van Slyke & Sendroy, "Macro" modification(3).
III " " " " " " " "Micro" " (3).
(a) performed by WRB, (b) performed by RHK with attention to details stressed by Eisenman(5).
IV Open Carius method of Wilson & Ball(4).
V Volhard method modified especially for milk, Method 1 of Sanders(7).
VI Analysis of 1:10 dilution by potentiometric method.
VII " " 1:10 " , deproteinized by tungstate method of Wu(13).

for a sensitive galvanometer because of its cheapness, ruggedness, and lower current drain, so that one or 2 cm of silver wire provide enough electrode surface. Such an instrument can be purchased; or a suitable one can be easily assembled from standard radio parts(2,pp.82-84).

Procedure for analysis. In preparation for a series of analyses, a large excess of chloride is added to the solution in the funnel. $AgNO_3$ is then added from the burette until the meter indicates the end-point. Since the solution in the funnel is now exactly neutralized, and its exact volume is immaterial (although small volumes increase sensitivity), analyses can begin. (a) The funnel is drained until 5 to 10 ml remain. (b) An aliquot (0.1 to 1.0 ml) of the specimen is pipetted into the funnel. (The neutralized solution already in the funnel facilitates rinsing of micropipettes calibrated "to contain.") (c) $AgNO_3$ is added from the burette until the meter again indicates the end-point. (d) The burette reading is mentally converted to the chloride concentration of the unknown in mEq/L by shifting the decimal point (and multiplying or dividing by 2 if necessary). Steps (a) to (d) are repeated for each succeeding analysis. If the end-point is exceeded, "Dilute Chloride Solution" can be added dropwise to neutralize the

TABLE II.
Results of Dilution of Unknown Solutions and Addition of Known Amounts of Chloride.

Nature of unknown	Conc. of unknown in final sol., % (v/v)	Added chloride, mEq/L	Chloride conc. of final sol. Mean ± stand. dev. (No. of titrations) Calculated mEq/L	Observed, mEq/L	Diff., mEq/L	Aliquot size, ml
Rat urine "D"	100	0	*	122 ± .7 (2)		.2
	50	0	61	60.4 ± .5 (4)	—.6	.2
	50	50	111	110.6 ± .5 (4)	—.4	.2
	50	100	161	160.5 ±1 (4)	—.5	.2
	20	0	24.4	23.5 ± .6 (4)	—.9	.2
Evaporated milk	100	0	*	65.4 ± .5 (6)		.5
	100	0	*	66.2 ±1.1 (4)		1
	80	80	132.6	131.7 ± .7 (3)	—.9	1
	40	160	186.3	186 ± .4 (2)	—.3	1
	4	160	162.6	162.7 ± .1 (2)	+.1	1
	1.2	80	80.8	80.6 ±0 (3)	—.2	1
	1.2	160	160.8	160.8 ± .1 (2)	.0	1
Beef serum "A"	100	0	*	101.1 ± .4 (17)		.5, 1
	80	80	160.9	160.3 ± .7 (5)	—.6	.5
	40	160	200.5	199.1 ±1 (5)	–1.4	.5
	10	0	10.1	9.9 ±0 (5)	—.2	1
	4	160	164	163.7 ± .6 (5)	—.3	.5
	1.2	80	81.2	81.2 ± .5 (5)	.0	.5
	1.2	160	161.2	160.4 ± .6 (5)	—.8	.5

* The other values in this column are calculated from the observed value in this row.

solution in the funnel before the titration is repeated. At the end of a series of analyses, accumulated precipitate is wiped off the glassware and indicator electrode; and the lower tip of the oxalate half-cell is capped by a rubber policeman, or simply reimmersed in fresh "Funnel Electrolyte," to prevent drying. Cleaning the indicator electrode occasionally with fine emery paper maintains its sensitivity.

Validation of the method. Interfering substances. Sufficient amounts of the following anions may be expected to interfere: bromide, chromate, cyanide, dichromate, ferricyanide, ferrocyanide, iodide, nitrite, thiocyanate and thiosulfate. Several mg or more of each of the following substances were added to the solution in the funnel at the end of a titration without producing any apparent effect on the end-point: acetone, ammonium carbonate, amytal, calcium nitrate, chloral hydrate, creatinine, dextran, ethyl alcohol, gelatin, glucose, inulin, lactic acid, paraldehyde, polyvinylpyrrolidone (PVP), sucrose, sulfathiazole, toluene, tryptophane, urea, urethane, uric acid and the following sodium salts: acetate, benzoate, bicarbonate, citrate, monobasic and dibasic phosphate, sulfate and tartrate.

Pure NaCl. The accuracy of the stoichiometric relationship is shown by titration of various volumes and concentrations of pure NaCl solutions (Table I). The mean values differ from the actual concentrations by less than 0.7% in each case.

Urine. Urine specimens from rats and man have been analyzed by standard procedures of the Volhard type(3-5), including hot digestion in the case of rat urine because of the protein normally present(6), and by this potentiometric method without any digestion (Table I). The results agree within 1%. Twenty-seven of these analyses were completed in an hour's time, except for the washing of the specimen containers. Analysis of a specimen of rat urine diluted various amounts, and with various amounts of NaCl and KCl added, showed satisfactory agreement with the concentrations calculated from the analysis of the original specimen (Table II).

Evaporated milk. When evaporated milk is added to the funnel electrolyte, a white floc appears in the clear liquid, and the titration can be performed without difficulty. Dilution and addition studies showed agreement within 0.7% (Table II). The "open Carius" method

of Van Slyke and Sendroy and some of its modifications(3-5), when applied to solutions of evaporated milk, gave results which commonly differed among themselves by 1 to 5% (Table I). Another modification of the Volhard method, designed especially for milk analysis(7, Method 1), also was distressingly inexact in our hands. The potentiometric method gave very consistent results, which differed from the reference values no more than the latter differed among themselves.

Serum. Upon the addition of serum, the solution in the funnel becomes opalescent, foam formation at the stirrer becomes troublesome, and the titration curve becomes much less steep. Added chloride was satisfactorily recovered (Table II), but the one sample of beef serum that has been studied carefully gave values 2% higher by direct potentiometric titration than by the other methods, which agreed closely among themselves (Table I).

Discussion. Effect of protein. Protein seems to interfere with some potentiometric chloride methods previously described(8), whereas its presence does not seem to impair the accuracy of some others(9). Probably these differences are due to the acidity of the solutions used (10,11) and the degree to which the protein is precipitated by the analytical conditions. In the present method, the small amount of protein in the urine of normal adult male rats (6) seems neither to precipitate, nor to cause error (Table I). Indeed, although large coagula of AgCl may form during the titration of purely inorganic solutions (impairing mixing), this is completely prevented by addition of a trace of gelatin to the solution in the funnel, without affecting the accuracy. In the analysis of milk, the prompt appearance of a white floc and the absence of frothing suggest that most of the protein is precipitated by the funnel electrolyte and causes no trouble. In the case of serum, the limited observations suggest that excessive amounts of protein remain in solution, causing the undesirable frothing and insensitivity, and combining with significant amounts of silver.

Accuracy. As an accuracy of 1% was considered adequate for the purposes for which this method was intended, commercial glassware was used without recalibration. In practice, considerably better precision has generally been obtained, and similar results can be expected in routine use. Indeed, precision comparable to the typical results presented in Tables I and II has been regularly achieved, after the first few hours' practice, by several other investigators who have adopted the method for their work. For urine, the absolute accuracy seems equally good. For milk preparations, the method is at least as accurate as the methods selected for comparison, and much more satisfactory. Although results tend to be higher than by the digestion procedures, the latter are known to be very susceptible to chloride loss(4,5,12). This is borne out by our experience that triplicate aliquots of a milk specimen digested simultaneously, although consistent, occasionally give very much lower values than other replications. The unsatisfactory nature of our initial experience with serum, compared with the satisfactory precision by other methods, discouraged us from further studies in this direction.

Summary and conclusions. 1. Modifications of Northrop's method of potentiometric titration of chloride are presented which retain its great convenience while eliminating interference from urates. 2. Analyses can be completed at the rate of 20 to 30 per hour, using aliquots of 0.1 to 1.0 ml, with a precision of better than 1%. 3. Accuracy of at least 1% for urine chloride determinations is evidenced by analysis of known solutions of NaCl, recovery of both KCl and NaCl added to urine specimens, and comparison of the values with those obtained by standard methods. 4. The method appears satisfactory for the analysis of evaporated milk preparations without modification: giving accurate recovery of added chloride, better consistency than reference methods, and as good agreement with them as their own variation (up to 5%) will allow. 5. The apparent unsuitability of the method for unmodified serum is discussed briefly.

It is a pleasure to acknowledge the encouragement of Dr. E. M. Landis and Dr. J. R. Pappenheimer, and

the assistance of the latter in the design of the vacuum-tube voltmeter used in these studies.

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Plasmal Reaction in the Blood and Hemopoietic Organs. (19869)

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Although the occurrence of the plasmal reaction of Feulgen and Voit(1) has been observed in many tissues and cells, no reference has been found in the literature concerning its presence in the cells of the blood and hemopoietic organs.

Material and methods. Ten adult normal rats of the Wistar strain were killed by a blow on the head. Smears of blood, bone-marrow, thymus, spleen and lymphatic glands were made and immediately immersed (before drying) in a mixture of equal parts of Schiff's reagent and saturated $HgCl_2$ solution during one minute, and then passed through 2 sulfurous acid baths of 5 minutes each and then rinsed with distilled water. Control smears were immersed in Schiff's reagent diluted with an equal part of distilled water for the same period, and afterwards treated as above described. Counterstaining with diluted methyl green solution was performed in some slides in order to help the identification of the cells. Smears from blood and sternal bone marrow obtained from 3 patients without hematological disorders were similarly treated. A positive reaction is indicated by a red stain present in the test slides and absent in the controls.

Results. As human and rat blood and bone marrow cells presented a similar reaction, the following data apply to both.

a) *Blood cells.* A positive reaction was observed in the cytoplasm of neutrophilic and eosinophilic granulocytes, monocytes and in the platelets. Reaction of neutrophils and monocytes was diffuse throughout the cytoplasm; eosinophils presented a less intense staining, localized between the generally unstained granules. Due to the small number of basophils no observations on them are reported. A positive reaction could be observed in the majority of the lymphocytes, sometimes diffuse throughout the cytoplasm and occasionally localized in granules. Red blood cells were always negative.

b) *Bone marrow cells.* The reaction was intensely positive in the cytoplasm of the

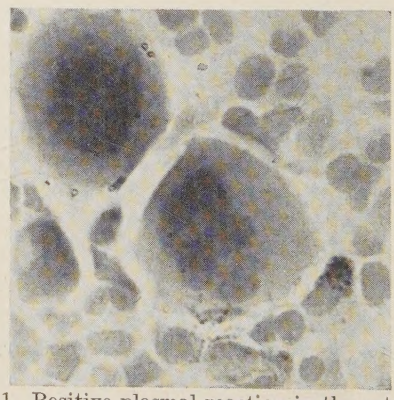

FIG. 1. Positive plasmal reaction in the cytoplasm of megakaryocytes and eosinophils. Nuclear counterstaining with methyl green. Approx. × 1400.

megakaryocytes. A weaker but undoubtedly positive reaction was observed in the cytoplasm of neutrophilic and eosinophilic myelocytes, plasmacytes and reticular cells. In the human material, cells which could be recognized as proerythroblasts and basophilic erythroblasts presented a juxtanuclear positively reacting zone. More mature forms did not stain.

c) *Thymus and lymphatic glands.* A positive reaction was observed only in the plasmacytes and reticular cells. Lymphocytes were always negative in these organs. In the plasmacytes the reaction was localized in a juxtanuclear zone, diffusely or in granules. Granulocytes when present reacted positively.

d) *Spleen.* A red stain denoting a positive reaction could be observed in the megakaryocytes, neutrophilic and eosinophilic granulocytes, macrophages, plasmacytes and reticular cells. These cells stained similarly as the above described ones.

Discussion. The positive plasmal reaction observed indicates the presence of plasmalogens (glyceryl-phosphoryl-ethanolamine and possibly analogous compounds (Lovern)(2) in the blood and hemopoietic organ cells. The positivity of the reaction in the cytoplasm of the megakaryocytes and in the platelets, adds more cytochemical evidence to the megakaryocytic origin of the platelets. The physiological significance of the presence of these compounds in the cells is unknown, but perhaps it might be connected with a protective mechanism(3). This reaction has been applied to pathological human material and a detailed account of the results obtained will be published elsewhere.

Summary. A positive cytoplasmic reaction was obtained by a modified technic of plasmal reaction in the following cells from human and rat blood and hemopoietic organs: megakaryocytes, platelets, neutrophils, eosinophils, lymphocytes, monocytes, plasmacytes, early erythroblasts and reticular cells.

Thanks are due to Prof. Junqueira for his helpful suggestions.

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Effect of Protamine Sulfate in Neutralizing Coagulation Defect Produced by Paritol.* (19870)

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In the constant search for an ideal anticoagulant, many new compounds with potent anticoagulant ability have been tried clinically. One of these new compounds is paritol, a polysulfuric acid ester of polyanhydromannuronic acid. Its mode of action has not been definitely determined but it is thought to be similar to that of heparin. Animal experiments(5) have suggested the presence of an antithrombin inhibitor in the peripheral blood as the natural inactivator of paritol.

The use of powerful anticoagulants always carries the possibility of hemorrhage. In view of this it is desirable to have an adequate neutralizing agent which will assure some degree of safety from hemorrhage. Protamine sulfate has been used as an antagonist to the coagulation defect produced by heparin. It seemed advisable to determine the extent of this neutralizing effect on the coagulation defect produced by paritol. Protamine sulfate was found(4) to have a clear-cut neutralizing effect upon paritol activity in dogs and rab-

* Abridgement of thesis submitted by Dr. Bartholomew to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

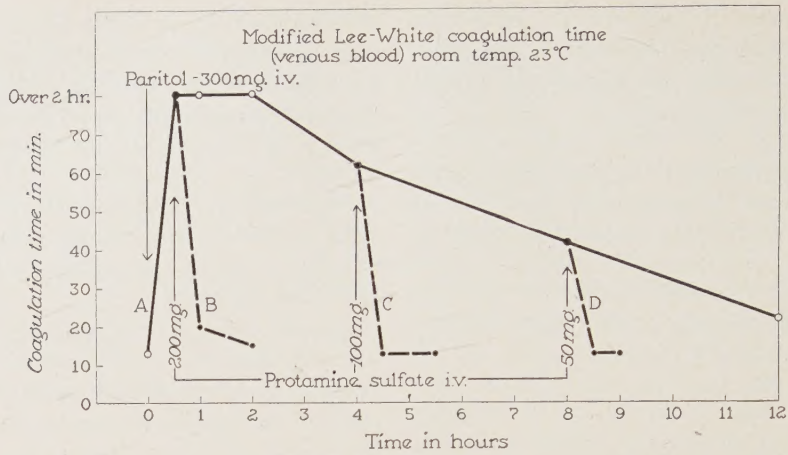

FIG. 1.

bits. The coagulation time of the blood in these animals returned to normal within 5 minutes after administration of protamine sulfate. If these results are directly applicable to clinical studies, then there would seem to be little risk of serious hemorrhage from such a powerful anticoagulant as paritol. There appears to be no report at this time on the neutralizing effect of protamine sulfate on the coagulation defect produced by paritol in human subjects.

Method of study. All subjects, 15 male and one female, had been hospitalized because of various types of vascular disease. The ages ranged from 21 to 82 years. The subjects presented no evidence of hepatic, renal, or hemorrhagic disease. In agreement with Sorenson and Wright(8) it was found that 300 mg of paritol injected intravenously produced and maintained a prolongation of the coagulation time of whole venous blood greater than 20 minutes for approximately 8 to 14 hours. Unless otherwise stated paritol† was injected intravenously in a dose of 300 mg throughout this investigation. Protamine sulfate was administered intravenously in a 1% solution. Immediately before the injection of paritol and for various intervals up to 24 hours after its administration the coagulation time was measured. When the coagulation time had returned to normal the subject was again given paritol. During this second period an attempt was made to study the effect of protamine sulfate in neutralizing the coagulation defect caused by paritol. After careful venipunctures had been performed the coagulation times were determined by a modified Lee-White method and by the Barker coagulochronometer(1). Since both methods produced similar results, the following discussion will refer to the modified Lee-White method. The average clotting time of the 16 subjects by the Lee-White technic was 12.5 minutes with a range of 11 to 16 minutes.

Results. The anticoagulant activity of paritol was rapid as evidenced by the coagulation defect produced within 30 minutes after its injection, and prolonged as noted by the 8 to 12 hours required for the coagulation time to return to normal (Fig. 1, curve *A*). An attempt was made to neutralize this coagulation defect when it was maximal and at various other intervals during its period of activity.

Fig. 1, curve *B*, illustrates the neutralizing effect of 200 mg of protamine sulfate administered 30 minutes after the injection of paritol. This is in striking contrast to the uninhibited anticoagulant effect of paritol as shown in curve *A*. Previous attempts with smaller amounts of protamine sulfate (100 to 150 mg) were not effective.

Fig. 1, curve *C*, is an example of the neutralizing effect of 100 mg of protamine sulfate upon the coagulation defect 4 hours after the administration of paritol. Smaller amounts

† Lot 432B supplied by courtesy of Wyeth, Inc., Philadelphia.

of protamine sulfate were found to be ineffective. Fig. 1, curve *D*, is an illustration of the response to 50 mg of protamine sulfate given 8 hours after the administration of paritol.

Comment. Protamine sulfate was found to exert a strong neutralizing effect on paritol activity. There were two substantial differences between its neutralizing effect upon paritol and upon heparin. Parkin and Kvale (3) found that the reaction between heparin and protamine sulfate was completed within 5 minutes. In the case of paritol it required a longer time. The other important difference was the large amount of protamine sulfate required to produce an adequate neutralizing effect upon the activity of paritol (Fig. 1, curves *B* and *C*). The use of such doses may possibly be hazardous because of the well-known toxic effects of protamine sulfate itself (2,6,7).

Two reactions occurred in our studies. Each began within 30 minutes after the injection of protamine sulfate during an attempt to neutralize the anticoagulant activity of paritol which had been given previously. They consisted of chills, fever, nausea, vomiting, burning epigastric pain and aching in the lumbar region of the back. These symptoms began to subside in a few hours and had disappeared completely within 4 days. The one unusual feature that was noted in these 2 cases was that the protamine sulfate had been administered in a more rapid manner than that recommended by other authors. This reaction did not occur when the protamine had been given 4 or 8 hours after the paritol had been given.

Summary. This study indicates that protamine sulfate is an active antagonist of paritol in the human subject. Larger doses than those used in neutralizing the effect of heparin are required to counteract the defect produced by paritol. Large amounts of protamine sulfate may produce toxic symptoms and therefore should be used with caution.

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A Colorimetric Method for Evaluating Chymotrypsin Inhibitors in Human Serum. (19871)

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Elevation of chymotrypsin inhibitor levels occurs in human blood serum with various pathological states(1), with pregnancy(2), and with certain types of mental disorders (3). Determinations of these inhibitor levels by the method of West and Hilliard(4), using homogenized milk as a substrate, can be used to follow the course of diseases such as cancer, and serve as a guide in evaluating various types of therapy(5-7).

Since the determination of chymotrypsin inhibitor levels by the method of West and Hilliard employs a nonsynthetic substrate (milk) and involves subjective end point determinations, it was decided to look for a simple synthetic substrate whose hydrolysis

could be determined colorimetrically. The method presented in this paper is based on the work of Iselin, Huang, and Nieman(8). These workers observed that hydroxamides of aromatic alpha amino acids are hydrolyzed by chymotrypsin and, since these hydroxamides give colored solutions in the presence of ferric chloride, that their hydrolysis by chymotrypsin may be quantitated by colorimetric analysis.

Materials and methods. Substrate. The synthetic substrate, l-tyrosine-hydroxamide, is prepared by the method of Cunningham, *et al.*(9) and is buffered to pH 7 with tris (hydroxymethyl) aminomethane hydrochloride acid buffer.* In a 100 ml volumetric flask, dissolve 100 mg of l-tyrosinehydroxamide in 0.1 M buffer with gentle heating. Cool, and bring to volume with buffer. This solution is stable. A mixture containing 0.5 ml of buffered substrate, 0.5 ml buffer, and 5.0 ml methanol-$FeCl_3$ solution gives a Klett reading† of approximately 250 (green filter No. 540, reference at 0 with distilled water). *0.1 M Buffer.* To 12.11 g of purified tris (hydroxymethyl)aminomethane and 22 ml of 4 N HCl in a 1000 ml volumetric flask, add approximately 900 ml distilled water. Adjust to pH 7.0 with 1 N HCl and bring up to volume. Filter this solution just before using. *Chymotrypsin.* Dissolve 20 mg crystalline chymotrypsin‡ in 20 ml of 0.1 M buffer. Shake the solution well to insure proper mixing and refrigerate for an hour before using. This enzyme solution must be prepared freshly and standardized daily. The activity of different lots of chymotrypsin will vary and it will decrease gradually even when stored at refrigeration temperatures. *Stock Ferric Chloride Solution.* Dissolve 7.0 g $FeCl_3 \cdot 6H_2O$ in 200 ml of 1 N HCl and bring up to a 250 ml volume with absolute methanol and filter. *Methanol-Ferric Chloride Color Reagent.* To 50 ml of stock ferric chloride solution add 250 ml absolute methanol. For rapidity in the procedure and to eliminate toxic effects of pipetting methanol, this solution should be added to the test mixtures from a 25 ml burette. *Blood Serum.* Tests must be made on fresh blood serum or on serum which has been refrigerated not longer than 24 hours. Hemolysis interferes with the accuracy of the test. Fasting blood samples are preferable.

Standardization of the chymotrypsin control. To each of 6 test tubes (for convenience in mixing, the tubes should be approximately 13 x 120 mm) add 0.5 ml substrate and then 0.5 ml of freshly prepared chymotrypsin solution. Mix quickly with a rotary motion and place in a 37°C water bath; agitate every 20 minutes. Incubate the first 2 tubes for exactly 1½ hours, and stop the reaction by adding 5 ml methanol-ferric chloride reagent. Mix and determine the density in a Klett colorimeter (filter 540, reference with distilled H_2O). The color develops immediately and is stable for several hours. If these first two enzyme control tubes check each other and give a Klett reading between 90 and 95, the 1½-hour incubation period is used for the test and the remaining four enzyme control tubes may be discarded. If the reading exceeds 95, the remaining tubes are allowed to incubate for longer periods in order to bring their colorimetric readings within the proper range. The increment of time beyond 1½ hours may be estimated by referring to Fig. 1 which shows the change in the density readings of the *chymotrypsin control* with time. The usual period of incubation is 2 hours.

Determination of chymotrypsin inhibitor in serum. Pipette 0.1 ml serum directly into the bottom of each of 4 test tubes. Add 0.5 ml substrate to each. To tubes 1 and 2, which will be the *serum-substrate controls,* add 0.5 ml buffer. To tubes 3 and 4, the *serum assay tubes,* add 0.5 ml standardized chymotrypsin solution. Immediately after adding the enzyme, mix the contents of all 4 tubes with a rotary motion, place in a 37°C water bath and record the exact time. These tubes should be swirled about every 20 minutes dur-

* Purified tris (hydroxymethyl) aminomethane may be purchased from G. Frederick Smith Chemical Co., Columbus, O., or the impure product obtained from Commercial Solvents, New York, may be prepared for use by twice crystallizing from absolute ethanol.

† The optical density is equal to the Klett reading multiplied by 0.002.

‡Crystalline chymotrypsin may be obtained from Spicer-Gerhart, Sunland, Calif., or from Armour Co., Chicago, Ill.

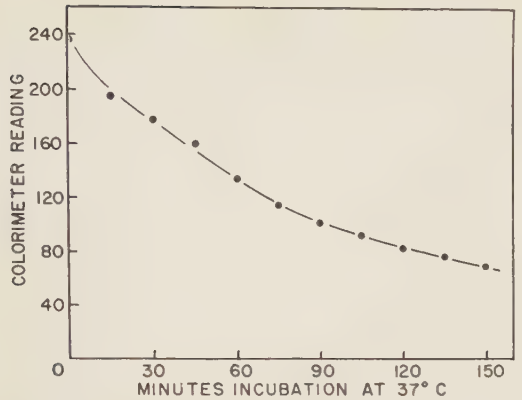

FIG. 1. Change in density readings of the chymotrypsin control with min of incubation in a 37°C water bath.

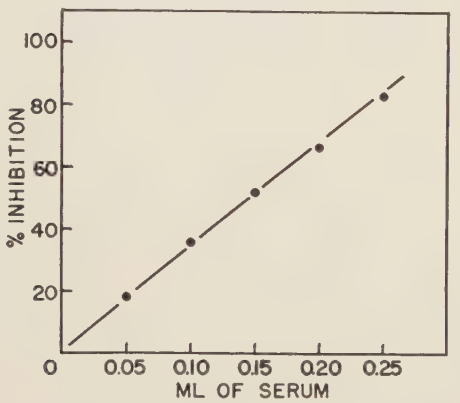

FIG. 2. Inhibition of enzyme activity with increasing amounts of serum.

ing the incubation period which has been determined by the chymotrypsin control as described above. At the end of this period, 5.0 ml of methanol-ferric chloride solution is added to all 4 tubes. This stops the reaction in tubes 3 and 4. *Density readings on all serum containing tubes must be made immediately, since a fine protein precipitate will form within a few minutes after the methanol-ferric chloride color reagent is added.* Therefore, when testing more than one serum a five minute interval should elapse between the addition of enzyme to each succeeding set of serum tubes, thus allowing time for colorimetric readings to be made on these 4 tubes before the incubation period has elapsed for the following set.

Calculations. The serum chymotrypsin inhibitor values, expressed in terms of per cent inhibition, are calculated as follows: % inhibition $= A - C/S - C \times 100$. A—Colorimeter readings for serum assay tubes; C—Colorimeter readings for chymotrypsin control; S—Colorimeter readings for serum-substrate control.

Discussion. Fig. 2 shows the linear relationship between enzyme inhibition and the amount of serum used in the assay. Under the experimental conditions described above, the sera from normal individuals give inhibitions ranging from 15 to 30%. The empirically determined curve in Fig. 3, based on data from 400 determinations,§ shows the relationship between units of chymotrypsin inhibitor as determined by the milk method (4) and inhibition of tyrosinehydroxamide hydrolysis. Tables I and II show the effect of varying either the enzyme or the substrate level in the test.

Summary. A colorimetric method of determining serum chymotrypsin inhibitor levels has been described. This method has the following advantages over the original milk method described by West and Hilliard: 1. It is based on the hydrolysis of a stable, synthetic substrate. 2. Colorimetric determina-

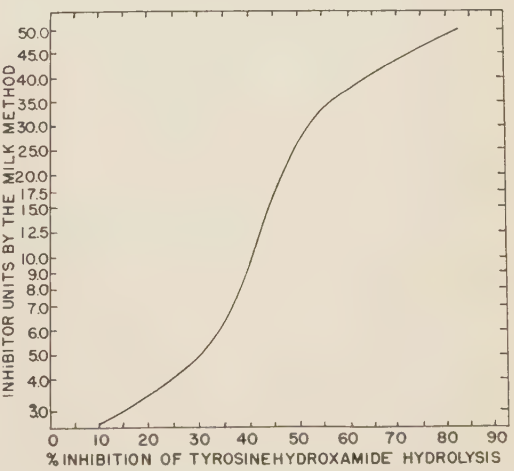

FIG. 3. Relationship between units of chymotrypsin inhibitor as determined by the milk method and the % inhibition of tyrosinehydroxamide.

§ The authors are indebted to Dr. Philip M. West and his laboratory staff at the Long Beach Veterans Administration Hospital for the data from which the graph in Fig. III was derived.

TABLE I. Effect of Varying the Concentration of Chymotrypsin Solution on Analysis of Serum Chymotrypsin Inhibitors.

Chymotrypsin sol'n, mg/ml	Incubation time, min	Klett readings: Serum substrate control (S)	Klett readings: Enzyme control (C)	Klett readings: Serum assay tube (A)	% inhibition
.25	120	248	180	244	94.1
.50	"	"	135	208	64.6
.75	"	"	98	161	41.5
1	"	"	83	129	27.9
1.50	"	"	52	90	19.4
2	"	"	40	63	11.1

TABLE II. Effect of Varying the Concentration of Substrate Solution on Analysis of Serum Chymotrypsin Inhibitors.

Substrate* sol'n, mg/ml	Incubation time, min	Klett readings: Serum substrate control (S)	Klett readings: Enzyme control (C)	Klett readings: Serum assay tube (A)	% inhibition
.25	105	86	32	49	27.5
.50	"	137	54	77	27.7
1	"	250	83	145	37.1
1.50	"	362	120	203	34.1
2	"	490	165	264	30.5

* l-tyrosinehydroxamide.

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Inhibition of Rapid Production of Antibody by Cortisone. Study of Secondary Response.* (19872)

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(Introduced by Robert F. Loeb.)

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The administration of cortisone or ACTH to rabbits results in a diminution of circulating antibody, as has been demonstrated by independent quantitative immunochemical studies(1,2). The diminished level of antibody resulted if the hormone was given during the period of primary immunization, or after antibody production was well established. Since the disappearance rate of rabbit antibody pas-

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† National Research Council Fellow in the Medical Sciences, 1951-53.

sively administered to rabbits was not altered by the administration of cortisone(2,3), it appeared that the lower amounts of antibody found in actively immunized animals could not be attributed to increased catabolism of antibody protein and that synthesis of antibody was inhibited. To study the effect of cortisone on the synthesis of antibody protein, it appeared desirable to employ a system in which the rate of synthesis is so very rapid that the rate of degradation may be considered negligible. Such a system obtains in the specific anamnestic or secondary response, in which the reexposure of an animal to a previously injected antigen is usually followed by an abrupt increase in circulating antibody to several times its former or basal level.

This study indicates that pretreatment with cortisone markedly inhibits the synthesis of antibody protein during the elicitation of the secondary response.

Experimental. The rabbits used in this experiment were from a group which, several months previously had been simultaneously immunized with crystallized egg albumin by 3 different procedures for other purposes. The rabbits labelled AV had been immunized with alum precipitated egg albumin administered intravenously; those labelled ES, with a saline solution of egg albumin intravenously; and those labelled AC, with alum precipitated egg albumin injected intracutaneously. After a rest period of 2 months, the animals were distributed into 2 groups, I and II, comparable according to method of prior immunization and antibody levels previously achieved. Equal distribution was further demonstrated by comparison of the antibody levels at 2 baseline periods (see *Results* and Table I). At the indicated intervals, all of the rabbits were subjected to the following sequence of procedures: 1) bleeding for first baseline levels (Day —12), 2) bleeding for second baseline level (Day —6), 3) intravenous injection of 1 ml of alum precipitated egg albumin containing 1 mg crystallized egg albumin nitrogen (Day 0), 4) first post-stimulus bleeding for antibody determination (Day +5), and 5) second post-stimulus bleeding for antibody determination (Day +13). Eight animals comprised Group I and received

TABLE I. Effect of Cortisone on Antibody Levels of Rabbits before and after Induction of a Secondary Response with Crystalline Egg Albumin.

Rabbit No.	Days before and after the antigen stimulus: −12	−6	+5	+13
	μg AbN/ml			
Group I—Treated with 25 mg cortisone daily from 4th day before antigen				
AV 168	52	32	28	24
169	83	65	48	68
177	48	35	17	—
ES 180	0	9	0	0
187	16	14	7	0
AC 193	37	39	33	—
195	9	6	7	—
199	19	—	25	—
Group II—Controls				
AV 170	12	9	50	128
171	73	53	100	140
ES 186	32	30	39	41
188	16	30	156	243
AC 192	2	2	22	37
202	38	27	59	144
204	17	8	15	98

cortisone; 7 animals comprised Group II and served as controls. Beginning on Day —4 and continuing for the remainder of the experiment, cortisone‡ was administered to the rabbits of Group I in daily dosage of 25 mg intramuscularly. Antibody analyses were carried out in duplicate by the quantitative precipitin method of Heidelberger and Kendall (4), employing the Heidelberger and MacPherson micromodification(5,6) when indicated by preliminary precipitin tests. When supernate tests or the ratio of antigen nitrogen to antibody nitrogen indicated that the desired degree of slight antigen excess was not present, repeated duplicate analyses were made to achieve optimal precipitation of antibody. The amount of anti-egg albumin is expressed as micrograms of antibody nitrogen per ml of serum (μg AbN/ml).

Results. Before the stimulating dose of antigen was given, the animals of Groups I and II were comparable in the distribution of their baseline antibody levels (Table I, Fig. 1). On Day —12 before the stimulating dose of antigen was administered, the range of titers in Group I was 0-83 μg AbN/ml with

‡ We are indebted to Merck and Co., Inc., Rahway, N. J. for the supply of cortisone acetate (Cortone).

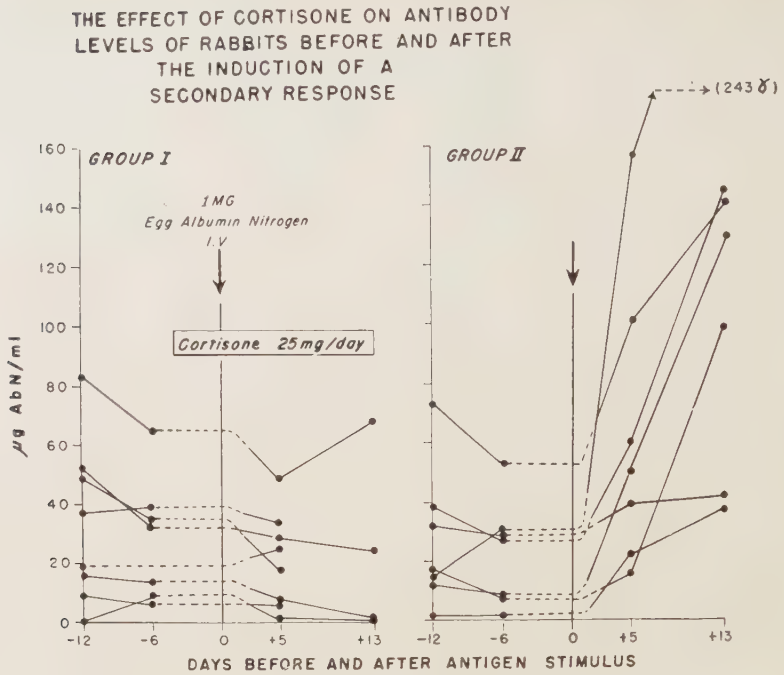

FIG. 1.

the median animals showing 19 and 38 μg AbN, while that of Group II was 2-73 μg AbN/ml with a median of 17 μg AbN/ml. Similarly, on Day —6, the range and the median of Group I were 6-65 μg AbN/ml and 32 μg/AbN/ml, respectively, and those of Group II were 2-53 μg AbN/ml and 27 μg AbN/ml, respectively.

Five days after the administration of 1 ml alum precipitated egg albumin containing 1 mg egg albumin nitrogen, a precipitous rise occurred in the antibody levels of the control animals of Group II, as seen in Table I and Fig. 1, with increases of severalfold in some instances. The range of absolute values was 15-156 μg AbN/ml with a median value of 50 μg AbN/ml. The antibody levels of the cortisone-treated animals of Group I remained approximately the same, or were slightly reduced, with a range of 0-48 μg AbN/ml and median values of 17 and 25 μg AbN/ml. By the 13th day after the antigen stimulus, further marked increases occurred in the control group with little change in the few remaining cortisone-treated animals. There was a substantial incidence of diarrhea and death in rabbits treated with 25 mg cortisone for several days. From previous experience, it appears unlikely that the effects of cortisone on antibody production are related to debilitation. Some animals with marked loss of weight and in morbid states, as well as well-nourished healthy animals, ranked haphazardly among both the better and poorer antibody producing groups(1).

Discussion. The present study, in conjunction with other work(1-3,7) indicates that cortisone or ACTH administration results in lower antibody levels. Failure to consider factors such as species, dosage, antigen-antibody system and method of analysis has contributed to some of the discrepancies noted in the current literature, as has been discussed(12). The lower antibody level is in large part due to an inhibition of the synthetic phase of antibody protein metabolism by cortisone, much as that hormone appears to inhibit synthesis of many tissue proteins, although some proteins may be effected in other ways(8-11).

Other steps associated with the production of antibody appear to be suppressed by cortisone. "Assimilation" of antigen, a necessary prelude to antibody synthesis, is apparently

inhibited as suggested by the work of Fagraeus and Berglund(13), and Moeschlin *et al.*(14). Employing an ingenious splenic transplant method, Fagraeus and Berglund demonstrated that cortisone inhibited the secondary response of rabbits to typhoid bacilli if the hormone was present shortly after the antigen injection. Two or 3 days after antigen stimulation, the presence of cortisone did not appear to inhibit antibody synthesis, as measured by the double dilution method. Morphologically, the site of "assimilation" of antigen is strikingly disturbed by cortisone in studies on acute disseminated encephalomyelitis where an absence of granuloma formation at the site of antigen-adjuvant deposition was noted (15). In addition are the reports of visible persistence of injected heterologous erythrocytes within the cells of cortisone-treated animals(16) and the many observations on the persistence and proliferation of various microorganisms in experimental and clinical infections of cortisone-treated hosts(17).

The production of antibody may in part be dependent on the stimulation of an inflammatory response(29) and the inhibition of antibody production by cortisone may be considered as part of the non-specific anti-inflammatory activity of cortisone observed in studies on wound healing and inflammatory processes(18). The examples of failure to assimilate antigen cited above may also be classified with this general action of the hormone. Clinically, the increased serum globulins and fibrinogen associated with various inflammatory states are also diminished coincident with the diminished inflammation resulting from cortisone or ACTH administration(19). There are, therefore, at least 3 steps in the formation of antibody at which cortisone probably exerts an inhibitory effect: the "assimilation" of antigen, the inflammatory reaction that is associated with injection of antigen, and the synthesis of antibody globulin. Perhaps these steps, in turn, have a common biochemical denominator.

Since several similarities with respect to lymphoid tissue and antibody production exist among vit. B_6 deficiency, X-radiation and adrenal cortical hyperactivity(20), it appears pertinent to note that the specific anamnestic response has also been inhibited by vit. B_6 deficiency in rats(20). With X-ray, Dixon *et al.* observed some delay in the appearance of the specific anamnestic response(21), but whether X-ray inhibits the magnitude of the response is not known since the amounts of antibody were not reported.

There has been considerable speculation on the allegation that non-specific factors, particularly adrenal cortical hormones(22) may elicit an anamnestic response. Repeated independent observations with quantitative immunochemical methods have failed to confirm any augmentation of circulating antibody due to the hormones(7,23,24) or to other non-specific stimuli(25-27). Indeed, following adrenal cortical hormone, a slight decrease in circulating antibody may be observed(7,24). Despite the great stimulus afforded by the specific antigen in the present study, the adrenal cortical hormone is decidedly inhibitory and cannot itself be considered a stimulus. There is no reason to abandon the concepts of Landsteiner(28) and of Heidelberger and his coworkers(25,26) that immunological reactions including the anamnestic or secondary response are chemically specific.

Summary. Cortisone markedly inhibited the rapid production of antibody associated with the specific anamnestic or secondary response. The effect of cortisone on antibody protein metabolism is discussed.

The authors are grateful to Dr. Elvin A. Kabat for his interest and advice.

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Role of Ionizing Radiation in Eliciting Tumors of the Pituitary Gland in Mice.* (19873)

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The finding that large fatal growths of the pituitary gland form in mice about a year after injection of thyroid-destroying doses of radioiodine, I^{131} (1), has been verified in several laboratories. These growths, as much as 200 times as large as the normal gland, are composed almost entirely of chromophobic cells, and their development is neither favored nor retarded by gonadectomy (4). Furth *et al.* (3,5) have found that transplants of the pituitary growths form large tumorous masses and metastasize in radiothyroidectomized hosts, and that later transplant generations grow even in normal hosts. More recently it has been shown that injection of thyroxine (2,6) or thyroid gland implants (6) inhibit the development of the tumors, suggesting that they are stimulated by the hypothyroidism induced by the radiothyroidectomy. However, radiothyroidectomy alone is insufficient to produce the tumors, since they do not develop in mice whose thyroids were destroyed by *small* quantities of I^{131} while they were being fed a low-iodine diet (6). Thyroids of mice fed such diets absorb a much larger proportion of the administered I^{131}, making it possible to destroy the thyroid glands with much smaller quantities of I^{131}. This suggests that the radiant energy in high doses of I^{131} may be a factor in precipitating the

* Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

TABLE I. Relationship Between Dose of I^{131}, Whole Body X-Irradiation, and Incidence of Late Hypophyseal Tumors.

No. of mice	Diet	Dose of I^{131}, μc	Dose of x-ray	Age at autopsy (mo)	Wt of pituitaries, mg — In detail	Avg	Median
25	Purina chow	0	0	14.2	1, 1, 1, 1.1, 1.1, 1.2, 1.3, 1.3, 1.4, 1.4, 1.4, 1.5, 1.5, 1.5, 1.5, 1.5, 1.5, 1.6, 1.6, 1.7, 1.7, 1.7, 1.7, 1.9, 2.1	1.46	1.5
21	Purina chow	200	0	13 –14.4	1.1, 1.7, 3.5, 8.4, 11.8, 22.2, 22.5, 23.5, 28.6, 30.6, 31, 51, 66.1, 81, 90.2, 100, 110, 112.5 Unweighed: 1 "small tumor," 2 "large tumors"	44.2	29.6
11	Purina chow	0	545r	13.6–14.7	.75, 1, 1.4, 1.6, 1.6, 1.7, 1.8, 1.9, 1.9, 2.2, 2.2	1.65	1.65
11	Low-iodine	30 + 170	0	13.6–15.3	9.7, 10, 21, 52, 99.2, 118, 157.1, 254.2 Unweighed: 1 "small tumor," 2 "large tumors"	103.3	34
10	Low-iodine	30	545r	13 –14.4	2, 12, 19, 24.4, 28, 41.9, 56.4, 80.2, 84.2, 151	49.9	35

tumorous development of the pituitary.

To test this possibility 77-day-old C_{57} male mice were placed on the Remington low-iodine diet for a period of one month before giving them 30 μc of I^{131}. This treatment has been found(6) to destroy or almost destroy (with no regeneration) the thyroids of C_{57} mice, but it is not followed by growth of hypophyseal tumors. Some of these animals (Table I) received a whole-body exposure of 545 r of whole body x-radiation (250 kvp: 30 ma 0.25 mm Cu, 1 mm Al; h.v.l. 0.9 mm Cu) 7 days after the I^{131} treatment. Others were given a high dose (170 μc) of I^{131} after the return to the normal Purina Lab Chow diet. As controls some mice, on a normal diet, were given the tumor eliciting dose of 200 μc of I^{131}, and others were given only the 545 r x-ray treatment. The organization of the investigation, the different types of control experiments, and the results are presented in detail in the table.

It seems clear that x-radiation is able to supply the necessary tumorigenic influence for the pituitary gland in mice whose thyroids have been destroyed by small amounts of I^{131}. The radiothyroidectomy or x-ray alone does not elicit hypophyseal tumors. It may be concluded that in the earlier experiments on production of hypophyseal tumors with large amounts of I^{131}, the endocrine (thyroidectomy), and radiant factors may have operated either additively or synergically in their tumorigenic action.

These experiments suggest that radiothyroidectomy induces physiological changes in the pituitary which sensitize the pituitary to the tumorigenic properties of the x-irradiation. It also is of interest that by this method of treatment, *i.e.* 30 μc I^{131} given to mice on a low-iodine diet, and followed by whole-body x-irradiation, tumors may be produced in a specific locus. To our knowledge similar instances of specific localization of a tumor following whole body x-irradiation have not been reported before.

Summary. 1. Pituitary tumors do not form after radiothyroidectomy of C_{57} mice by small

amounts of I^{131}. 2. Pituitary tumors result when such radiothyroidectomy is followed by whole-body x-irradiation, or by larger amounts of I^{131}. 3. Similar doses of x-rays alone do not lead to growth of the tumors. It appears that ionizing radiation induces these atypical growths in pituitaries physiologically altered by radiothyroidectomy.

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A Mechanism of Cholesterol Deposition on Arterial Walls. (19874)

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It is established that cholesterol deposition takes place in the arterial walls of animals (1,2) and man(3,4) and that such deposition is a forerunner of arteriosclerosis and atherosclerosis(5-7). The cause of this cholesterol deposition has not been known(8).

In view of the fact that at least some correlation exists between progressing age and cholesterol deposition in arteries, and in view of the working hypothesis that the fundamental aging reaction is a protein cross-linkage or tanning reaction(9,10) it seemed pertinent to determine whether known cross-linking agents for protein would render arterial wall surfaces selectively adsorptive to cholesterol.

Methods. To this end fresh hog aortas* were slit open. Round test pieces of 20 mm diameter were punched out by means of a cork borer. These test pieces were placed in water solutions of a cross-linking or tanning agent, and control pieces from the same aorta were placed in water. After an interval as shown below, all of these test pieces were immersed in a cholesterol suspension prepared as follows: Fifty g of cholesterol were milled on a rubber mill with submicron particle size acetylene carbon. The object of this was to color the cholesterol particles so that they could be followed visually and photographed. The rubber mill effects a very intimate mixture and complete wetting of the carbon particles by cholesterol. This was verified by microscopic examination. On subsequent dispersion in water, it was found that the carbon particles remain inside the cholesterol particles, and thus are believed not to affect the surface behavior of the cholesterol particles. The cholesterol thus pigmented was added to 300 g of a 4% solution of carboxy methyl cellulose in water, dispersed 10 minutes in a Waring Blendor, and diluted with an additional 200 cc of water. The resultant cholesterol dispersion is quite stable, and can be stored at least for several weeks, though it may be advisable to shake it before use to ensure uniformity.

The aortas were kept immersed in this cholesterol suspension for the periods of time shown below, whereupon they were placed in a 4 inches high by 3⅜ inches wide cylindrical jar containing 100 ml of clean tap water and washed twice by shaking for ½ minute in a shaking machine, ⅛ inch stroke, 450 cycles per minute. The areas which had been in contact with the cross-linking solutions showed heavy cholesterol deposits, while the other areas failed to adsorb cholesterol, and after washing as just stated, were completely free from any traces of this substance. Black

*I am indebted to Oscar Meyer Co. for their cooperation in supplying the aortas fresh from the packing line.

TABLE I. Effect of Certain Cross-Linking Agents on Cholesterol Adsorption by Hog Aorta.

Cross-linking agent	%	Time of immersion in cross-linker	Time of immersion in cholesterol dispersion	Color after wash
Chromic sulfate	10	10 min	10 min	B*
	10	1	10	B
	10	15 sec	10	B in spots only
	1	14 hr	14 hr	B
	.5	14	14	W
Mercuric chloride	10	14	14	W
	1	14	14	W
Lead acetate	3.1	48	10 min	B
	.3	24	10	B
	1	15 min	10	S
	.1	14 hr	10	W
Lead nitrate	.2	14	10	B
	.06	14	10	W
Copper sulfate	5	16	10	B
	1	16	10	S
	.5	16	10	W
Cobalt sulfate	5	16	10	B
	2	16	10	B
	.5	16	10	W
Formaldehyde	37	48	10	W
	3.7	48	10	W
Control, tap water		48	16 hr	W
		24	10 min	W
		12	10	W
		15 min	16 hr	W

* B = black; W = white; S = spotty.

color, when occurring, was due to the carbon inside the suspended cholesterol particles.

Results are summarized in Table I.

It is apparent from this table that *in vitro* the intima of fresh hog aortas may be made to adsorb and retain cholesterol from a carbon-pigmented aqueous cholesterol suspension. This effect is produced by treatment with certain cross-linking agents, of which lead acetate is one of the most efficacious, but copper and chromium salts are also potent. This is not a simple heavy metal effect, for mercuric chloride did not produce this effect even on the drastic test of 48 hours immersion in a saturated solution of mercuric chloride.

Fig. 1 illustrates the sharpness of the adsorption effect obtained with the lead acetate and chromic sulfate, and the total absence of

TABLE II. Reversal of Cholesterol Receptivity of Hog Aorta.

Treatment for attempted reversal of cholesterol receptivity induced by min. effective amount of agents shown in adjacent columns	Exposure time (min)	Chromium sulfate	Cupric sulfate	Cobalt sulfate
Ammonium iodide saturated	10	W†	W	Almost B
Ethylene diamine tetraacetic acid[1]* 10%	10	W	W	B
Choline chloride 2%	10	B	—	—
Disulfhydryl propanol[2] 10%	10	B	B	B
Pepsin .1%[3]	30	Almost W	Almost W	B
Trypsin .1%[4]	30	W	W	B
Ficin .1%[5]	30	W	W	Almost B
Papain .1%[6]	30	Almost W	Almost W	Almost W
Control	0	B	B	B

* [1] Eastman Kodak. [2] BAL in oil, Westcott and Dunning. [3] pH 2, 39°C. [4] pH 8, 39°C. [5] pH 5, 39°C. [6] pH 5, 39°C.

† W = white; B = black.

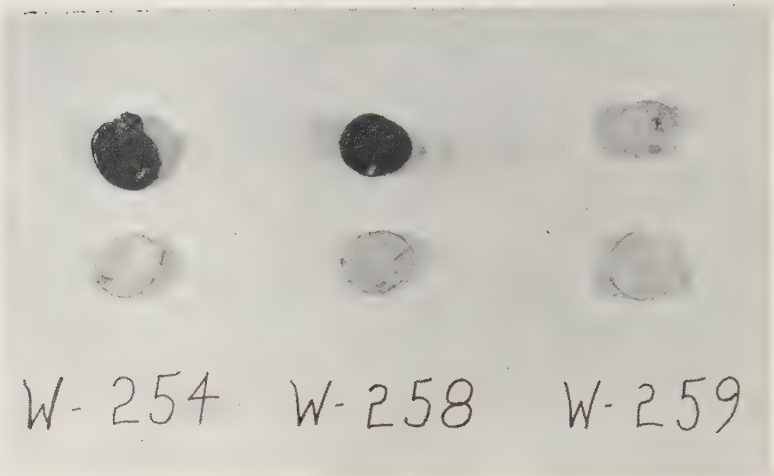

FIG. 1. Representative specimens from Table I. The lower row represents the controls in each case. W254 was treated with chromic sulfate 10% and W258 with lead acetate .3%. W259 was treated with saturated mercuric chloride for a period longer than 10 min and then immersed in the carbon-stained cholesterol suspension and washed.

visible adsorption after treatment with mercuric chloride, or by the untreated controls.

It may be pertinent here to refer to reports that cholesterol adsorption has been induced *in vivo* by uranium salts, which are known as effective protein-tanning agents(11).

It seemed of interest now to determine whether the cholesterol receptivity obtained by the protein cross-linking agents could be reversed. To this end sections of hog aorta were immersed in the cross-linking solutions shown in Table II under conditions which would make them receptive to the cholesterol. The minimum concentration and exposure time to cross-linking agent, shown in Table I, to give cholesterol adsorption, were employed. They were then immersed in treatments which it was felt might reverse the receptive conditions, and then were immersed in the cholesterol test dispersion, washed and observed. Black color of the specimens indicated that the treatment had not reversed the cholesterol receptivity, while white color of the specimens showed that these had been reversed from a cholesterolophilic to a cholesterolophobic condition.

It is seen from this tabulation that the receptive condition brought about with chromium or copper sulfate can be reversed with ethylenediamine tetraacetic acid, ammonium iodide, or proteolytic treatment, but that this is not effective for cobalt sulfate. Disulfhydryl propanol ("British anti-lewisite") is not effective under the conditions tested. Further work is in progress.

These results may justify the working hypothesis that the primary cause of cholesterol deposition and subsequent pathology in arteries may be a chemical process involving the protein material in the lining of the arteries, which changes this from a cholesterolophobic to a cholesterolophilic condition, and that this change may be due to a gradual cumulative action of certain protein cross-linking agents. It is certainly extremely suggestive that the loss of elasticity of the arterial wall, and its ability to adsorb cholesterol, may be related by so simple an hypothesis as the chemical change in proteins induced by cross-linking or tanning agents.

Summary. Certain substances reactive with proteins have been found *in vitro* to cause selective adsorption of suspended cholesterol to the inner lining of hog aorta.

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Microdetermination of Calcium in Blood Serum by Direct Titration. (19875)

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The microdetermination of calcium in serum by rapid titration with a chelating reagent, ethylene diamine tetracetate(1), has obvious advantages. In the method of Sobel and Hanok(2), in which the dye, eriochrome black T, is used as indicator, magnesium is also titrated, and the end-point is obscured in icteric and hemolyzed serum. Greenblatt and Hartman(3) propose a method with murexide (ammonium purpurate) as indicator, but do not mention interference by magnesium. Although they present data to show that the results obtained in serum, urine and spinal fluid agree with those obtained by a standard method, the end-point has been found so poor in this laboratory that we have not been able to titrate serum with the naked eye. We also have evidence(4) that it is not possible to titrate calcium in urine in this way.

In this paper we describe a method for the microtitration of calcium in serum in a photoelectric colorimeter with ethylene diamine tetracetate and murexide as indicator. Magnesium is not titrated in this method, and neither hemolyzed nor icteric blood interferes in the titration. In a later paper we will describe a macromethod for the titration of calcium in urine.

Experimental. We have used a Lumitron photoelectric colorimeter with an adaptor to hold tubes of 15 mm diameter. The solutions were titrated in Klett-Summerson microcolorimeter tubes while the tubes were in the colorimeter, and the colorimeter was read continuously during titration. Although this arrangement has been found most convenient, any colorimeter with an adaptor to hold tubes of this size, and any short tube of about 15 mm diameter should be suitable. A convenient burette was made by sealing with De Khotinsky cement a 4 inch hypodermic needle of about 18 gauge bore to the tip of a 0.2 ml Kahn pipette graduated in thousandths. The needle was bent at a right angle so that at least 3 inches of needle was perpendicular to the pipette, and the point of the needle was filed off straight. A piece of rubber tubing to serve as a reservoir for mercury was attached to the other end of the pipette, and the open end of the tubing was plugged with a glass rod. This burette was mounted on a horizontal wooden stand which also carried a screw clamp to propel the mercury and titrating fluid, and another bent needle which delivered nitrogen gas from a tank to mix the solution in the tube during titration. Our stand was so constructed that the entire burette assembly could be raised and swivelled at the start and at the end of each titration at the colorimeter.

Reagents. 1) Concentrated stock calcium standard solution. Dissolve 2.497 g of dry calcium carbonate with a minimal amount of hydrochloric acid. Neutralize with sodium hydroxide, and dilute to 500 ml. 2) Working standard calcium solution. Dilute 5 ml of the concentrated stock solution to 100 ml. 100 ml = 10 mg Ca. 3) Saturated picric acid

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(according to Folin) in water. 4) Murexide-sodium chloride indicator mixture. Grind 200 mg of murexide (ammonium purpurate) and 100 g of sodium chloride to a fine powder, and mix thoroughly. 5) Stock borate solution. Dissolve 62.0 g of boric acid in freshly boiled water containing 124.5 ml of 4.00 N sodium hydroxide, and dilute to 1 liter. 6) Borate buffer solution. Add 80.5 ml of 4.00 N sodium hydroxide to 400 ml of the stock borate solution, and dilute to 1 liter with freshly boiled water. 7) Concentrated sequestering reagent, S.E.D. Dissolve 4.0 g disodium ethylene diamine tetracetate in 800 ml of water containing 10.7 ml of 2 N sodium hydroxide. 8) S.E.D. reagent A. Dilute the concentrated S.E.D. reagent so that 0.5 ml of working standard calcium solution requires 0.100 ml of S.E.D. reagent A by method 1. 9) S.E.D. reagent B. Dilute the concentrated S.E.D. reagent so that 0.2 ml of working standard calcium solution requires 0.100 ml of S.E.D. reagent B by method 2.

Methods. Two methods are proposed. In method 1, which requires 0.5 ml of serum, the proteins are removed with picric acid and calcium is titrated in the presence of the yellow color of picrate. In method 2, 0.2 ml of serum is titrated directly, and a little caprylic alcohol is added to inhibit foaming in the alkaline solution which contains protein. Either method is suitable for icteric or hemolyzed serum, but method 1 is less affected by the abnormal color of serum and yields sharper end-points when hemolysis is excessive.

Method 1. Add 0.5 ml of serum with stirring to 2.0 ml of saturated picric acid in a centrifuge tube. After standing for a few minutes, add 1.5 ml of water. Mix thoroughly by inversion on parafilm, and let stand 10 minutes. After centrifugation, transfer 3.0 ml of the clear supernatant fluid to a microcolorimeter tube. Add 1.0 ml of borate buffer solution and about 90 mg of indicator mixture from a measuring tube(5). Place the tube in the colorimeter, which has been adjusted to 100% transmission with water in a second tube. Swing the burette into place, attach to the nitrogen and adjust the flow of gas to a maximum that yields steady readings. Titrate with S.E.D. reagent A with filter 580 until the end-point is reached, which is indicated by constant readings on the galvanometer. (The readings start at about 40% transmission, change slowly as the end-point is approached, and finally remain unchanged with increasing amounts of S.E.D. reagent at the end-point, which is about 22% transmission.) The concentration of calcium in the sample is calculated by the equation, Ml S.E.D./0.100 x 10.0 = mg % calcium.

Method 2. Add 0.2 ml of serum to 3.0 ml of water in a microcolorimeter tube. Mix, and add 1.0 ml of borate buffer and 90 mg of the indicator mixture. Finally add a small drop of caprylic alcohol, and place the tube in the colorimeter. Adjust the nitrogen as in method 1 and titrate with filter 580 in place, using S.E.D. reagent B. In this method the readings also start at about 40% transmission and become constant at the end-point, which is at about 22%. The calculation is also the same, Ml S.E.D./0.100 x 10.0 = mg % calcium.

The titrations with sequestering reagent were conducted at a pH of about 11.5 (glass electrode) since we have found that magnesium does not interfere in the titration of calcium in this region. Whereas the effect of magnesium is considerable at pH 10.5, concentrations as high as 20 mg % of magnesium do not affect the results at the higher pH. Fig. 1 shows the results obtained at pH 11.7 of a series of titrations in standard solutions of calcium in picric acid by method 1 in the presence and in the absence of 5 mg % of magnesium. Similar results have been obtained at pH 11.7 by method 2 in the absence of picrate.

We have tested both methods 1 and 2 in about 100 samples of serum in which calcium was also determined by a standard method (6) by precipitation as oxalate and titration with permanganate. In a representative series of 20 samples of serum, in which the calcium concentration varied between 5.4 and 13.9 mg %, the results determined by method 1 deviated between −0.6 and +0.4 mg % from those obtained by the standard method, and showed an average deviation of 0.24. The results determined by method 2 deviated

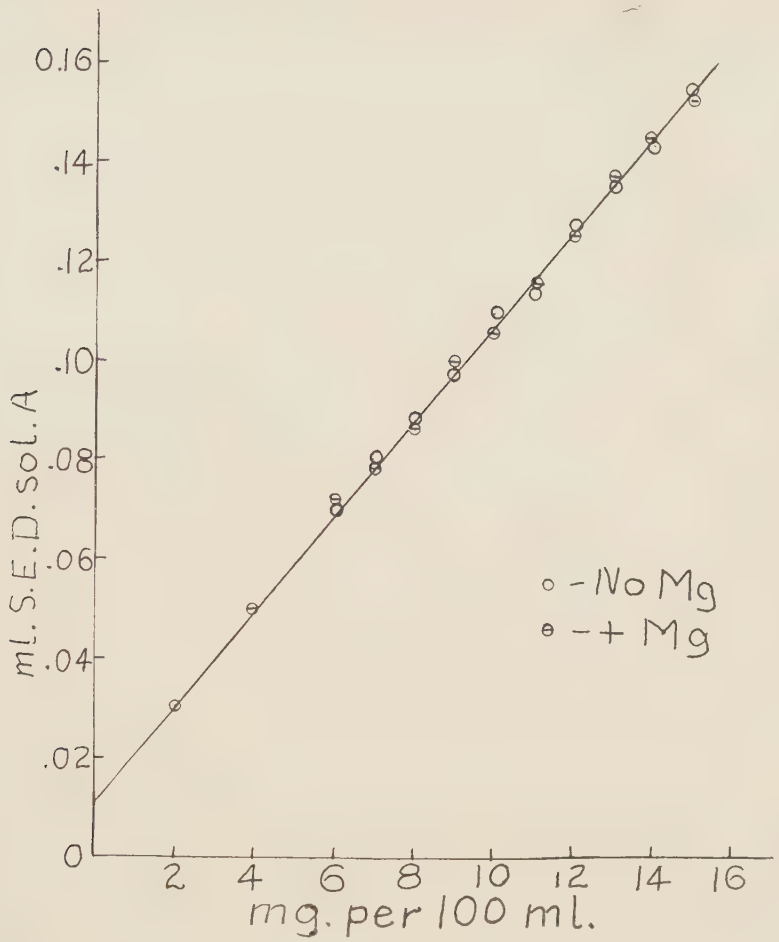

FIG. 1. Values of the titres of S.E.D. reagent A for varying conc. of calcium by method 1 in the presence and in the absence of 5 mg % of magnesium.

between –0.5 and +0.3 mg % from the values obtained by the standard method, and showed an average deviation of 0.20.

Summary. Methods are described for the microdetermination of calcium in serum by titration with ethylene diamine tetracetate in a photoelectric colorimeter at pH 11.6 with murexide as indicator. It is shown that magnesium does not interfere with the titration of calcium at this pH, and that the endpoint is reached when the readings on the galvanometer become constant. In method 1, the proteins are removed from 0.5 ml of serum with picric acid and the titration is conducted in a solution of picrate. In method 2, 0.2 ml of serum is titrated directly in the presence of protein. Data are presented to show that the results of both methods agree quite well with those obtained by precipitation as oxalate and titration with permanganate.

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Action of Fluoride and Other Reagents on Phosphorylation in Malignant and Certain Normal Tissues. (19876)

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The suggestion of Warburg(1,2) that tumors derive a large proportion of their energy for growth from anaerobic processes has stimulated inquiry into the mechanism of energy liberation and transfer in both tumor and normal tissues. It has recently been shown that enzymes of the tricarboxylic acid cycle occur in tumors (see the articles by Potter(3), Weinhouse and collaborators(4,5), and Olson(6), including reviews of literature. See also Schneider and Hogeboom(7) for literature regarding normal and cancer tissue mitochondria). Potter and Lyle(8), Clowes and Keltch(9), Williams-Ashman and Kennedy(10), and Kit and Greenberg(11) found that oxidations carried on by such enzymes from tumors can generate high-energy phosphate bonds which can be used for synthesis of cellular structural elements(10,11). It has also been shown by LePage(12,13), by LePage and Schneider(14), and by Clowes and Keltch(9,15) that the supernatant fraction remaining after the particulate elements of tumor and normal tissue have been centrifuged off can utilize the energy from glycolysis to synthesize organic phosphate compounds. The nature of the glycolytic reactions involved is now under study by Groth, LePage, Heidelberger, and Stoesz(16).

The object of the present investigation is to form a more quantitative estimate than has hitherto been made of the relative importance of anaerobic and aerobic processes in energy liberation in one tumor, the Walker 256 rat carcinoma, as compared to normal rat liver and brain.

Results. The methods used were those of Clowes and Keltch(9) as adapted from those of LePage and of Potter, except for the order in which the reaction components were mixed. In the present experiments the inorganic phosphate was a part of the mixture placed in the main compartment of the Warburg vessel and the reaction was started by addition of the tissue fractions from the side arm after equilibration with the gas phase; in the previous experiments(9) the tissue fractions were put into the main compartment and the reaction started by addition of phosphate from the side arm. Tissues were obtained and homogenized as described before(9). Particles were obtained by centrifuging at 20,000 g for one-half hour at 4°C, washing, and resuspending, as previously described. The reaction mixture contained the same components in the same concentration as before, unless otherwise stated. The substrate in all experiments was hexose diphosphate (HDP) and pyruvate.

Effect of fluoride concentration on phosphorylation by particulate and supernatant fractions. One of the principal difficulties in measurements of phosphorylation in cell-free fractions is the high phosphatase activity which appears as cells are broken up; this activity can be to some degree suppressed by the inclusion of fluoride in the incubation medium(3,17,18). The effects of fluoride upon aerobic and anaerobic phosphorylation by homogenates, particles, and supernate fractions were found to depend very sharply upon its concentration (Table I). The point to be stressed is that phosphorylation by the particles *increased* with addition of fluoride up to the optimum concentration of 0.0033 to 0.015 M. Optimum phosphorylation by the glycolytic system of the supernatant fractions was obtained when fluoride was omitted entirely. When the optimum rates of phosphorylation obtainable with homogenates or particles by adjustment of fluoride concentration were compared, it was found that the ratio of aerobic to anaerobic phosphorylation was greatest in liver and least in tumor, with brain intermediate between the other two. The phosphorus uptake of a given supernate was approximately the same in air or nitrogen and was lowered to a corresponding extent by any given concentration of fluoride. These results confirm and extend the observations

TABLE I. Effect of Fluoride on Phosphorus Uptake by Homogenates of Rat Liver, Brain, and Tumor and by the Particulate and Supernate Fractions Derived Therefrom by Centrifuging at 20000 g for 30 Min. Particles were washed once with buffered KCl, recentrifuged, and resuspended in buffered KCl. Incubation time, 40 min at 25°C. Substrate for both aerobic and anaerobic experiments was hexose diphosphate and pyruvate. Gas phase in aerobic experiments was air, in anaerobic experiments nitrogen. Each experiment based on material derived from 125 mg original tissue, except in the case of tumor particles, where material from 500 mg original tissue was used. All results expressed in μM.

Final molar conc. fluoride	Liver		Brain		Tumor	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
			Homogenate			
.0	14.7	−1.2	15.9	6.9	4.3	3.2
.0017	16.6	.8	16.5	5	8.8	7.2
.0033	17.1	2.1	17	7.3	13.3	10.6
.0067	17.5	3.3	17.3	8.7	14.2	10.8
.013	17.5	3.4	17.5	8.5	13.6	10.7
.02	17.3	3.1	17.3	8.1	12.8	10.5
.026	16.6	3.6	16.9	7.9	13.2	10.8
.033	16	2.6	15.9	7.8	12.8	10.7
.04	15	3.4	15.5	7.8	12.1	10.5
			Particles			
.0	4.6	−2	0	−1.1	−2.8	−1.2
.0017	10.5	−2.5	2.3	−1.6	−2.4	−1.1
.0033	14.1	−1.2	5.1	−1.2	− .8	− .5
.0067	13	−1.3	6.3	.5	0	.3
.013	12.4	− .5	8.4	.8	3.3	2.8
.02	10.8	− .6	7.5	.7	2.9	2.3
.026	8.5	− .4	7.6	.7	2.5	2.5
.033	7.7	−1.3	6.9	1	1.9	2.1
.04	6.1	0	6.3	.9	2.1	2
			Supernate			
.0	8.9	11.1	18	17.1	17.3	17.9
.0017	5.7	7.6	17.2	14.7	17.3	16.8
.0033	5.7	7.5	16.6	14.4	17.3	16.9
.0067	5.1	6.7	16.9	13.5	13.6	14.6
.013	5.1	6.2	13.5	11.7	8.3	10.5
.02	5.4	6.1	13.1	10.5	8.5	11
.026	5.6	6.4	13.1	9.7	8	11.5
.033	5.4	6.7	11.8	9.7	8.5	11
.04	5.3	6.2	12.2	9.3	7.6	11

previously reported(9,15).

Under carefully controlled conditions phosphatase activity of particulate elements from a mouse tumor is smaller when the particles are prepared in 0.25 M sucrose than in 0.15 M KCl(19). In the present experiments, brain or tumor particles prepared and tested in 0.25 M sucrose did not take up more phosphate than those prepared and tested in KCl; P uptakes in micromoles were: brain particles in sucrose, 10.8; in KCl, 14.6; tumor particles in sucrose, 5.7; in KCl, 5.0.

Effect of anaerobiosis on phosphorylation by homogenates and by particulate and supernatant fractions. As another attempt to assess the relative importance of anaerobic and aerobic processes in the 3 types of tissues, phosphate uptake and lactate production by homogenates, by particles, and by supernatant fractions of liver, brain, and tumor were measured (Table II).

The anaerobic and aerobic uptakes of inorganic phosphate by liver, brain, and tumor homogenates from 125 mg tissue were respectively, in micromoles, as follows: liver: anaerobic 2.3, aerobic 16.6; brain: anaerobic 9.0, aerobic 17.2; tumor; anaerobic 10.2, aerobic 13.1. Thus, phosphate uptake by the Walker 256 tumor homogenates under these experimental conditions was chiefly based on anaerobic reactions, that by liver chiefly on aerobic reactions, confirming the conclusion reached on the basis of measurements on the particulate and supernatant fractions(9).

The question arises whether competition may exist for phosphate between the anaerobic

TABLE II. Phosphorus uptake (P) and lactate production (L) by homogenate, particulate, and supernate fractions of liver, brain, and tumor as a function of amount of original tissue from which each fraction per vessel is derived, as a function of presence or absence of DPN, and as a function of presence or absence of DNC. Fractions were prepared and measurements made as described in Table I and text. Incubation time 40 min. at 25°C. Results in micromoles.

	Tissue equiv. per vessel, mg	Conc. DPN, moles/l	Conc. DNC, moles/l	Liver						Brain				Tumor					
				Homog.		Part.		Super.		Homog.		Part.		Super.		Homog.		Part.		Super.	
				P	L	P	L	P	L	P	L	P	L	P	L	P	L	P	L	P	L
In air	250	.0002	0	17.5	4.6	16.7	1.2	9.2	12.6	19.2	7.6	9.8	2	18.4	10.8	18.2	14.2	2.2	2.8	19	16
		0	0	17.3	.7	16.2	.9	1.9	2.8	18.8	4.4	.3	1.1	—	—	5.5	3.4	-.1	1.1	—	—
		.0002	.0005	.8	7.7	—	—	—	—	8.9	9.8	-.1	1.8	—	—	14.1	15.1	1.4	2.7	—	—
	125	.0002	0	16.6	4.1	10.2	.5	4.7	7.2	17.2	6.5	5.8	1.5	10	7.8	13.1	11.2	1.2	1.8	8.5	8.7
		0	0	15.5	.9	9.1	.6	1.2	1.4	2.4	2.2	.5	.5	1.7	2.1	2.9	1.4	-.2	.3	-.8	1.1
		.0002	.0005	2	5	-.5	.6	5.2	7.1	8.9	8.8	.7	1.4	12.3	9.9	9.9	9.4	—	—	10.5	9.4
	62.5	.0002	0	12.1	3.8	4.7	.6	—	—	16.6	4.7	3.2	1.6	5.8	4.8	6.6	5.5	—	—	—	—
		0	0	6.7	.8	3.9	.5	—	—	.7	1.1	-.2	1.2	—	—	.4	1.1	—	—	—	—
		.0002	.0005	1.9	3.8	0	.5	—	—	5.1	5.9	—	—	—	—	5.6	5.5	—	—	—	—
In nitrogen	250	.0002	0	2.1	9.1	-.2	1.5	8	12.1	10	9.9	-.7	2.7	18.2	11.7	14.6	18.9	1.6	3.4	17.4	16.9
		0	0	-4.8	.9	-1.1	1.4	.9	3.4	1.6	6.2	-1.9	1.2	—	—	-1.2	3.4	-1.3	1.6	—	—
		.0002	.0005	1	7.7	—	—	—	—	8.9	9.8	-.1	2.3	—	—	14	17.1	1.5	3.1	—	—
	125	.0002	0	2.3	6.2	0	.3	5.6	8.1	9	8.6	.9	1.8	9.8	9.8	10.2	11.2	—	—	8.7	10
		0	0	-4.3	.6	-.5	.4	2.1	2.8	1.4	3.4	-.1	1.4	2.4	3.7	.5	2.6	—	—	-1.3	1.5
		.0002	.0005	1.5	5.9	-.8	.6	5.7	7.1	8.8	9.6	.8	2.2	12	9.7	9.9	11.5	—	—	9.9	9
	62.5	.0002	0	3.2	4.3	.5	1.1	—	—	6.2	5.4	.5	1.3	6.1	4.7	5.4	5.7	—	—	6	6.1
		0	0	-1.2	.8	.1	.6	—	—	.3	3.2	—	—	—	—	.8	2.2	—	—	—	—
		.0002	.0005	1.6	3.5	0	.9	—	—	6.1	5.2	—	—	—	—	6.3	5.8	—	—	—	—

and aerobic systems, such that the actual phosphate uptake in oxygen occurring via glycolysis may be much less than the anaerobic values of 2.3, 9.0, and 10.2 for liver, brain, and tumor, respectively; *i.e.*, that there is a pronounced Pasteur effect. The anaerobic and aerobic lactate productions for the same series from 125 mg liver, brain, and tumor homogenates were, respectively, in micromoles, as follows: liver: anaerobic 6.2, aerobic 4.1; brain: anaerobic 8.6, aerobic, 6.5; tumor: anaerobic 11.2, aerobic 11.2, thus indicating a moderate Pasteur effect for liver and brain but not for tumor. If the lactate figures for 250 mg of original tissue are compared, liver, brain, and tumor all show a moderate Pasteur effect, that of the liver being greatest. In a previous communication (9) it was noted that with brain and liver but not with tumor the combined lactate production of the individual particle and supernate fractions was considerably larger than that of the homogenate. This was interpreted as a Pasteur effect. In the experiments reported in Table II the lactate production of particles plus supernate greatly exceeded that of homogenate in the experiments with liver and brain and also in the experiment in which 250 mg of tumor tissue was employed, but with 125 mg of tumor tissue the combined lactate production of particles and supernate was less than that of homogenate. It would appear from the above results that a moderate Pasteur effect is observed in the case of all 3 tissues, greatest in liver, intermediate in brain, and least or absent in tumor tissues.

One question which was not completely settled in previous publications(9,15) was whether or not the particulate elements could effect phosphorylation under anaerobic conditions with HDP and pyruvate as substrate. It has now been found that when the particles are washed virtually free of phosphate and kept separate from the glycolytic substrates until the equilibration with nitrogen has been completed, there is no significant phosphorus uptake by liver and brain particles and sometimes no significant uptake by tumor particles under anaerobic conditions. For example, the phosphorus uptake under aerobic and anaerobic conditions of liver particles was 16.4 and

TABLE III. Phosphorus Uptake, Lactic Acid Production, and Oxygen Consumption by Particulate and Supernate Fractions of Liver, Brain, and Tumor in Air and in Nitrogen. Each experiment is based on material derived from 250 mg original tissue, except in the case of the tumor particles, where material from 500 mg original tissue was used. For other experimental details see Table I and text. All results expressed in μM.

Fraction	Liver			Brain			Tumor		
	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake
				In air					
Particles	16.4	.6	6	11.3	2.5	3.6	3.4	1.5	2.3
Supernate	9.2	12.6	1.1	18.4	10.8	1	19	16	—
				In nitrogen					
Particles	—.2	1.5	—	.3	2.4	—	.7	5.8	—
Supernate	8	12.1	—	18.2	11.7	—	17.4	16.9	—

—0.2, of brain particles 11.3 and 0.3, and of tumor particles 3.4 and 0.7 (Table III). The phosphorus uptake of the supernates was, as previously observed, the same under aerobic and anaerobic conditions: in the case of liver 9.2 and 8.0, in the case of brain 18.4 and 18.2, and in the case of tumor 19.0 and 17.4. Furthermore, the lactate production in all supernates was virtually the same under aerobic and anaerobic conditions. It appears that the phosphorylation by tumor particles under anaerobic conditions which was previously mentioned may have arisen, at least in part, from traces of oxygen which were present during the 10-minute equilibration period with nitrogen, particularly at the beginning. It is possible that the particles in the intact cell may have a system capable of glycolysis, in addition to the nonparticulate glycolytic system dealt with here.

On account of the relatively high fragility and low content of particles in the tumor tissues, larger amounts have frequently been employed in the hope of obtaining significant results. This makes it more difficult to wash the particles free from supernate. It is possible, therefore, that the small phosphorylating effect sometimes observed when tumor particles are used under anaerobic conditions may be due to occlusion of small amounts of the water-soluble, anaerobic, glycolytic, phosphorylating mechanism. This question is being further investigated with other tumors.

The absolute rates of phosphorus uptake by the homogenates were found to depend upon the amount of tissue used and upon addition of diphosphopyridine nucleotide (DPN) to the medium; with the homogenates under aerobic conditions great dependence upon DPN addition was found with tumor and brain and relatively little with liver. The same was true with particles, confirming other investigators(10,19). Omission of DPN led to a marked depression of phosphate uptake by the supernatant fractions of all 3 tissues, being about the same under both aerobic and anaerobic conditions. For example, the anaerobic P uptakes in micromoles per 40 minutes with and without addition of 0.0002 M DPN were (all for 125 mg tissue): liver supernate, 5.6 and 2.1; brain, 9.8 and 2.4; tumor, 8.7 and —1.3 (Table II).

Dinitrocresol (DNC), 5×10^{-4} M, did not significantly inhibit phosphorylation by homogenates of brain and tumor under anaerobic conditions (Table II) and may therefore be used as a reagent to test for the fraction of the phosphate uptake under aerobic conditions (*i.e.*, in an atmosphere of air or O_2) which is dependent on glycolytic reactions. In the case of liver homogenates, the anaerobic phosphate uptake of which is relatively low, the addition of dinitrocresol under anaerobic conditions sometimes causes further lowering of phosphorus uptake.

Efficiency of phosphorylation under anaerobic conditions. Formation of one mole of lactic acid from hexose diphosphate under the experimental conditions employed should lead to the uptake of one atom of inorganic phosphorus. To determine the actual ratio, lactate formation and phosphorus uptake were measured for the supernate fractions as a function of the incubation time, fluoride concentration, and presence or absence of added hexokinase (Table IV). The optimum values of the

TABLE IV. Phosphorus Uptake and Lactic Acid Production by Supernate Fractions of Liver, Brain, and Tumor, in Relation to Incubation Time, Addition of Excess Hexokinase, and Presence or Absence of Fluoride. Gas phase, air. Each experiment based on material derived from 250 mg original tissue. For other experimental details see Table I and text. All results expressed in μM.

	No yeast hexokinase added				Yeast hexokinase added			
	With fluoride		Without fluoride		With fluoride		Without fluoride	
Incubation time (min)	P uptake	Lactate formed	P uptake	Lactate formed	P uptake	Lactate formed	P uptake	Lactate formed
				Liver				
5	2.4	3.3	−.2	3.1				
10	3.3	4.9	1	5.8				
20	5.1	7	−.5	7.8				
40	7.3	11.4	.5	14				
				Brain				
5	3.6	1.8	3	1.6	10.7	7	14.9	12
10	5.3	3.3	4.9	4.2	14.8	9.7	19.7	14.4
20	8.8	4.5	6.6	5.5	—	—	—	—
40	10.2	6.5	9.1	8.4	14.3	11.1	20.4	23.1
80	16.6	11.8	—	—	13.6	11.8	20.4	24.2
				Walker 256 tumor				
5	2.8	2.8	2.1	1.8	6.4	6.1	12.2	12.2
10	3.2	4.2	2.8	3.6	6.9	6	16.5	16.5
20	5.8	6.4	3.2	6.5	—	—	—	—
40	7.9	9.4	4.8	8.8	6.7	8	17.3	18.3
80	12.7	10.8	7.2	14.6	8.2	10.9	17.3	20.7

ratio of phosphorus uptake to lactate formation were found to be as follows: liver 0.7, 20 minutes incubation, fluoride present, hexokinase absent; brain 1.5, 10 minutes incubation, fluoride present, hexokinase either present or absent; tumor 1.2, 10 minutes incubation, fluoride present, hexokinase present. In the absence of added yeast hexokinase, lactate formation and phosphorus uptake follow an approximately linear course for the first 40 minutes of incubation. With added hexokinase the reaction under the experimental conditions employed was virtually complete after 10 minutes. The limiting factor in the overall phosphorus uptake was, therefore, the rate at which the high-energy phosphate could be transferred to a stable form. The reactions responsible for the phosphate uptake in excess of that accounted for by lactate production have not been identified; they may be the same as those recently described by Groth, LePage, Heidelberger, and Stoesz(16).

Iodoacetate and phosphate uptake. It was mentioned above that only aerobic phosphorylation is suppressed by dinitrocresol, that associated with glycolysis being left unaffected (Table II). The effects of various concentrations of iodoacetate, which can combine with sulfhydryl groups(20) of enzymes such as phosphoglyceraldehyde dehydrogenase (21) and those of the tricarboxylic acid cycle (22), were determined upon both particulate and supernatant fractions (Fig. 1). Both aerobic and anaerobic phosphorylations were suppressed by iodoacetate.

Discussion. It now appears that the particulate elements isolated from tumors can generate high-energy phosphate bonds by oxidation of substrates of the tricarboxylic acid cycle(3-10,19). However, since the tumor materials employed have invariably contained a certain amount of normal tissue, the possibility remains that the small oxidative phosphorylating effect observed may be derived from that source. There is no doubt that the supernate fractions can generate such high-energy phosphate bonds by glycolytic reactions(9,12-14). From the published results now available it appears that generation of high-energy phosphate bonds in the Walker 256 tumor takes place largely at the expense of glycolytic rather than oxidative reactions, whereas generation of such bonds in liver is largely based on aerobic reactions. Particulate elements of other tumors may have a relatively greater capacity for aerobic phosphorylation than the Walker 256 tumor (10,19).

In this and preceding papers(9,15) the

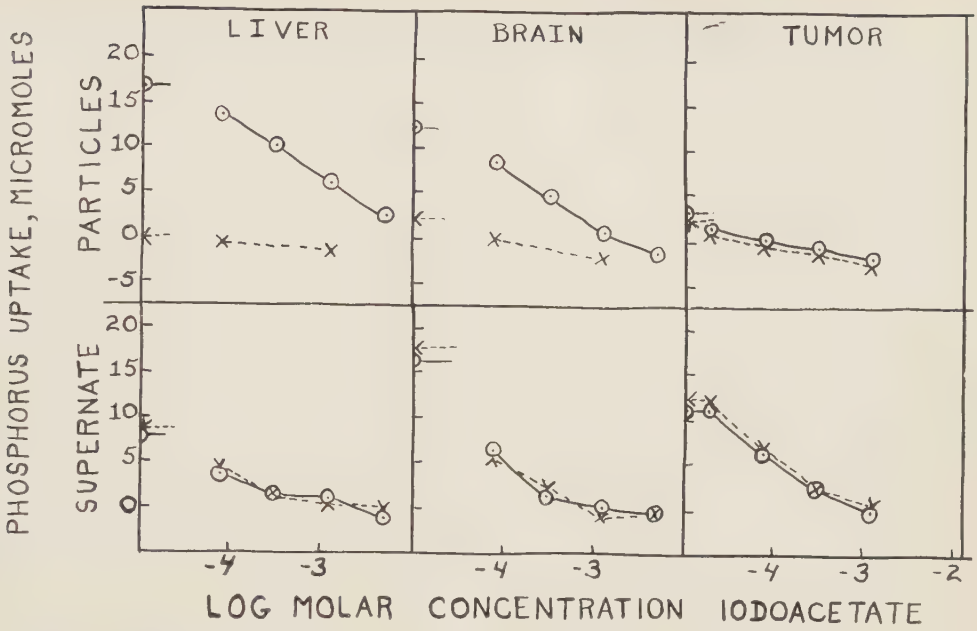

FIG. 1. Effect of iodoacetate on phosphorus uptake by particulate and supernate fractions of liver, brain, and tumor: ○———○, in air; ×- - - -×, in nitrogen. Respective control values in air and in nitrogen designated by horizontal lines perpendicular to the ordinates. For other experimental details see Table I and text.

sensitivity to inhibitors of the particulate and supernatant fractions of tumor has been measured and compared with that of the same fractions from normal tissues. It was found that aerobic phosphorylation by particles of liver and brain was inhibited by dinitrocresol or by calcium ion and that phosphorylation in the supernate fractions of liver, brain, and tumor was relatively insensitive to these reagents. The only difference between the normal and tumor fractions with respect to the action of these agents was that the tumor particles exhibited a slight residual phosphorylation even in the presence of DNC or calcium chloride. These observations have been confirmed in the present investigation. However, as it is difficult to obtain enough tumor particles to permit the extensive washing employed with the liver and brain particles, it is possible that the DNC-resistant phosphorylation of the tumor particles may arise from occlusion of supernate in the tumor particulate fraction. This possibility requires further investigation.

In the present paper the effects of fluoride and iodoacetate on homogenates, particulate fractions, and supernate fractions from the same tissues have been determined. While aerobic phosphorylation is increased and anaerobic phosphorylation is decreased by addition of fluoride, no significant difference between the sensitivity of the tumor and the normal fractions has been found.

Although the phosphorylation by the cell-free fractions from the tumor drops markedly when DPN is omitted from the medium, this dependence on added DPN is not unique to the tumor; similar decreases in phosphorylation by fractions from brain and to a lesser degree from liver were observed upon omission of DPN.

Since, with the exceptions noted, the tumor tissue appears to differ only quantitatively and not qualitatively from the 2 normal tissues, the high anaerobic phosphorylation cannot, at least for the present, afford an explanation of the rapid growth of tumor relative to normal tissues.

Summary. The effects of fluoride, of anaerobiosis, and of iodoacetate upon esterification of phosphate by homogenates, by particles, and by supernatant fractions of liver, brain, and tumor have been determined. The requirement for DPN by the various fractions has also been investigated.

1. Phosphorylation by the homogenates or particles increases with addition of fluoride up to an optimum concentration of about 0.01 M; optimum phosphorylation by the supernatant fractions is obtained when fluoride is omitted entirely. By this adjustment of fluoride concentration the preponderance of glycolytic over oxidative phosphorylation in the Walker 256 tumor is even more marked than has been previously recognized. 2. Under the present experimental conditions phosphorylation by the particulate fractions of liver and brain and of tumor within the range of experimental error was found to be dependent on aerobic processes, while that in the supernatant fractions was completely dependent on anaerobic reactions. The maximal values obtained with the supernate fractions under anaerobic conditions for the ratio of phosphorus uptake to lactate formation were: liver, 0.7; brain, 1.5; tumor, 1.2. 3. Phosphorylation by the particulate and supernate fractions was markedly inhibited by iodoacetate; no significant differences between the sensitivity of the tumor fractions and that of the liver or brain fractions was observed. 4. Phosphorylation in the Walker 256 tumor appears to differ from that in normal tissues principally in quantitative rather than in qualitative respects.

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Mucolytic Enzyme Systems XIX. Comparison of Hyaluronidase Inhibitor and Heparin Levels in Serum.* (19877)

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Because evidence was obtained suggesting that heparin might be a component of the non-specific hyaluronidase inhibitor in blood serum(1), determination of the degree of parallelism between the serum concentrations of heparin and inhibitor was investigated. For this purpose rabbits were subjected to two experimental procedures which had been reported to change the heparin or antithrombin concentration of the blood, and measurements of heparin and inhibitor levels as a function of time were carried out. The first procedure was suggested by the finding of Volkert(2) that the elevation in antithrombin activity of blood resulting from peptone shock could be inhibited by blocking the reticuloendothelial system with India ink. Accordingly, the effect of peptone shock with and without India ink injections was studied. The second procedure was suggested by the report of Bell and Stuart (3) that desoxycorticosterone alleviated "hyperheparinemia" in a clinical case. The simultaneous effect of this hormone on the heparin and inhibitor concentrations in the blood was measured.

In addition to these two *in vivo* studies, the *in vitro* effects of certain phenazine, oxazine and thiazine dyes, and also of aureomycin, on the heparin and inhibitor content of blood serum were determined because Haley and Stolarsky(4) showed that the dyes in question counteracted the anticoagulant action of heparin *in vitro,* and Waisbren and Glick(5) showed that aureomycin caused a decrease in heparin concentration in human plasma *in vitro.* The influence of the dyes and aureomycin on testicular hyaluronidase was determined, incidentally.

Methods. The procedures used for obtaining the rabbit serum, storage in the deep-frozen state, and the viscosimetric assay for hyaluronidase and its non-specific inhibitor have been indicated previously(6). Details of the assay(7,8), as well as of the mode of expressing the percent inhibition(9), may be found in earlier papers of this series. The rabbits served as their own controls in the *in vivo* experiments since comparisons were made on the same animals before and after the treatments. For each analysis of the serum inhibitor, 0.025 cc of serum was used; however in the dye and aureomycin experiments 0.040 cc of serum was employed to magnify the effect of these agents on the inhibitor. The dye solutions were adjusted to pH 6.7 with 0.2 N NaOH before addition of the serum, and the aureomycin glycinate used was neutralized with 0.1 N HCl to pH 7.3. *Heparin* was determined by the method described by Jaques, Monkhouse and Stewart (10), which was based on an earlier study of Jaques *et al.*(11). The principle of the method is spectrophotometric measurement of the metachromatic color produced by the reaction of heparin with azure A, after purification of the heparin by precipitations with octylamine and brucine. As used in this study, certain details have been modified to sharpen the analytical precision as follows: To each 5-10 cc sample of serum, 1 cc 7% octylamine hydrochloride (Sharples) was added, mixed, and after a few minutes centrifuged at 1700 times gravity and –2°C for 15 minutes. The decreased temperature and increased time and speed compared to the original method made for more complete precipitation of the heparin complex. (For some human sera, particularly those from patients with nephrosis, heating at 56°C for 30 minutes before addition of the octylamine resulted in more

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TABLE I. Effect of Peptone Shock on Hyaluronidase Inhibitor and Heparin Concentrations in Rabbit Serum.

Treatment and time of sampling	No. of rabbits	Mean % inhibition	Mean μg % heparin
Before peptone inj. (1 g/kg) i.v.	7	24 ± .5	230 ± 8
.5 hr after peptone inj.	7	35 ± 1.4	480 ± 15
24 " " " "	6	32 ± 1.6	220 ± 16
72 " " " "	5	28 ± 2	230 ± 19
Before India ink inj. (.5 cc/kg) i.v. and 3 wk after end of above exp.	6	20 ± 1.1	220 ± 9
.5 hr after India ink inj.	6	19 ± 2.6	210 ± 37
24 " " " " and just before peptone inj.	6	17 ± 1.1	210 ± 10
.5 hr after peptone inj. (1 g/kg)	6	11 ± 1.6	130 ± 15
24 " " " "	6	18 ± .8	210 ± 10
72 " " " "	6	22 ± .3	200 ± 12

TABLE II. Effect of Intramuscular Injection of Desoxycorticosterone Acetate on Hyaluronidase Inhibitor and Heparin Concentrations in Rabbit Serum.

Treatment and time of sampling	No. of rabbits	Mean % inhibition	Mean μg % heparin
Before 1st inj. (25 mg DOCA in 5 cc sesame oil per day for 4 days)	10	16 ± .4	220 ± 2
Before 4th inj.	10	19 ± 1.2	310 ± 29
.5 hr after 4th inj.	10	25 ± 2.5	350 ± 34
2 " " " "	9	22 ± 1.4	290 ± 42
72 " " " "	10	23 ± 1.4	280 ± 29
120 " " " "	9	18 ± 1.3	250 ± 27
Before 1st inj. (5 cc sesame oil/day for 4 days) and 3 wk after end of above exp.	6	16 ± 0	250 ± 27
Before 4th inj.	6	18 ± 1	280 ± 20
.5 hr after 4th inj.	6	17 ± .5	280 ± 7
2 " " " "	4	17 ± .9	280 ± 0
24 " " " "	5	19 ± 1.3	300 ± 20
72 " " " "	6	17 ± .8	220 ± 39

complete precipitation. This was not true for the rabbit sera.) The treatment of the heparin complex followed that given in the original procedure(10) to the point of addition of phosphate buffer to the alkaline hydrolysate of the heparin-brucine precipitate. At this point 1 cc of 0.15 M KH_2PO_4 and 1 cc of water were added to the 2 cc of heparin in 0.05 N NaOH, the solutions were centrifuged for 10 minutes at about 15,000 times gravity to remove cloudiness that was occasionally encountered, 1 cc of azure A solution was introduced and the pH was checked with a glass electrode. If the pH was not 7.3 it was adjusted to this value. Usually not more than a drop of 0.05 N NaOH was sufficient for this purpose. Somewhat greater color development occurred at this pH than in more alkaline solutions. The azure A (National Aniline, 92% dye content) employed was made up to a 100 mg% stock solution every 2 months and it was diluted 1:9 before use. After addition of the azure A the optical density was measured at 530 mμ with a Coleman spectrophotometer. Blank and standard determinations were run in which physiological saline solution or heparin (Hynson, Westcott, and Dunning) solutions of known concentration were substituted for the serum. A linear relationship between optical density and amount of heparin was found over the range 10-60 μg, and the serum samples were adjusted to yield an amount of heparin in this range. Satisfactory recoveries of heparin added to serum were obtained.

After the present studies were almost completed, a report appeared by Monkhouse and Jaques(12) in which they applied to plasma the method of Homan and Lens(13) of separating heparin from protein by phenol, and a

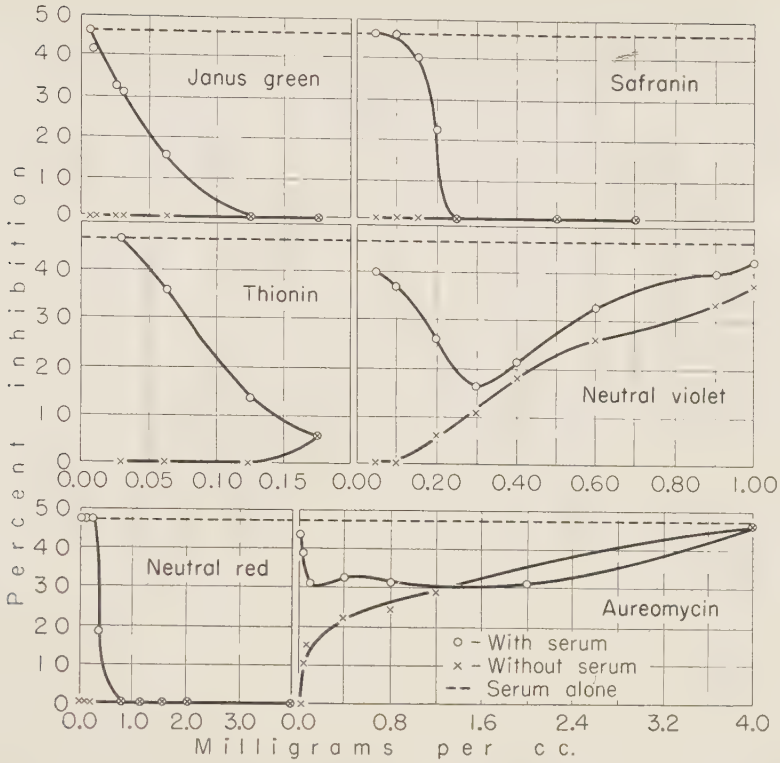

FIG. 1. Effect of dyes and aureomycin on the activity of non-specific hyaluronidase inhibitor of serum and testicular hyaluronidase.

further modification of the method was described later by Gibson *et al.*(14) who employed zinc hydroxide for the reprecipitation of the heparin.

Peptone shock experiments were carried out by administering single doses of 10% peptone (Merck) in 0.9% NaCl to the rabbits via ear vein and withdrawing blood at intervals as indicated in Table I. The peptone solution was freshly prepared, boiled for 10 minutes, cooled, and adjusted to pH 7.4 before injection by sterile technic. In a second group of experiments India ink (Higgins) was administered to the same animals after a period of recovery, subsequently they were given another peptone injection, and blood samples were taken at the intervals indicated in Table I. Administrations of desoxycorticosterone acetate in sesame oil ("Percorten", Ciba), and control injections into some of the same animals with oil alone, were conducted as indicated in Table II; the blood samples were withdrawn for analysis at the intervals given. In the *in vitro* dye and aureomycin experiments the ranges of concentration of these substances that were employed are given in Fig. 1. In all cases the solutions were added to the serum before other components of the reaction mixture.

Results. From the data in Table I it is apparent that the peptone shock elicited a significant maximum elevation of the serum concentrations of both the inhibitor and heparin within 0.5 hour. The heparin concentrations fell to the preinjection level after 24 hours but the inhibitor level dropped more slowly and was still slightly elevated after 72 hours. India ink injection had no perceptible effect on the heparin concentration but caused a slight fall in the inhibitor, and subsequent peptone shock produced a decrease in both of these factors within 0.5 hour (instead of the rise observed from the same degree of shock without previous ink-block) with a return to the preshock level within 24 hours.

A degree of parallelism was found in the rise of the inhibitor and heparin concentrations following desoxycorticosterone injec-

tions, Table II. A maximum was found, 0.5 hour after the fourth injection, while practically no effect was observed in the control experiment with sesame oil alone.

The data in Fig. 1 show that janus green, safranin, thionin, and neutral red have no direct effect on hyaluronidase activity under the conditions employed. Inhibition of the enzyme was obtained with neutral violet and aureomycin. However, all of the dyes reduced the effect of the serum inhibitor. In the case of neutral violet and aureomycin this latter effect was counteracted and, at higher concentrations, overcome by direct inhibition of the enzyme. With respect to concentration required to inhibit the serum inhibitor, janus green and thionin were more effective than safranin, neutral violet or neutral red.

Discussion. The present data indicate a general parallelism in the direction of the changes that have been made to occur in the heparin and inhibitor, but correlation of magnitude and time of change was somewhat variable. This might be due, at least in some degree, to the nature of the heparin analysis which can include other acid polysaccharides in addition to the heparin. The rise in heparin concentration that was observed following desoxycorticosterone injections contrasts with the finding of Bell and Stuart(3) that the hormone reduced the level of heparin or heparinoid substances in the blood of a patient with "hyperheparinemia" as measured by clotting tests and protamine titration. On the other hand clinical evidence, such as that given and cited by Margulis(15) and Smith *et al.*(16), that particular hormonal or stress stimulations of the adrenal result in a decrease in clotability of blood in some cases may be related to the present data as well as to the elevation in the level of the inhibitor in serum that these adrenal stimuli also produce(6).

The data obtained by *in vitro* experiments with the basic dyes, along with data previously obtained with toluidene blue(8), show that the dyes most effective in reducing the activity of the serum inhibitor are those which Haley and Stolarsky(4) found most effective in reducing the anticoagulant action of heparin in rabbit plasma. Thus, Janus green, thionin, and toluidene blue, which had similar effects on the inhibitor, were more effective for a given dye concentration than safranin, neutral violet and neutral red.

The earlier data of Waisbren and Glick(5) on the role of aureomycin in decreasing heparin concentration *in vitro,* and lowering coagulation time when administered *in vivo,* is supplemented by the present data showing that the antibiotic also reduces the serum inhibitor *in vitro.*

Summary. The effects of peptone shock (with and without block of the reticuloendothelial system by India ink), and administrations of desoxycorticosterone on the nonspecific hyaluronidase inhibitor and heparin concentrations in the blood serum of rabbits at intervals before and after the treatments were observed. A general parallelism was noted in the direction of the changes produced in both serum factors, but variations were observed with respect to the magnitude and time of change. *In vitro* experiments were carried out on the effect of janus green, thionin, toluidene blue, safranin, neutral violet, neutral red, and aureomycin on the serum inhibitor and on hyaluronidase. A general correlation was observed between the anti-heparin action of these substances and their anti-inhibitor effects.

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Motile Life of Bovine Spermatozoa in Glycine and Yolk-Citrate Diluents at High and Low Temperatures. (19878)

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In experiments with sea urchins and other invertebrates it has been found(1,2) that the addition of any one of a number of amino acids or peptides to the spermatozoa, diluted in sea water, extends very greatly their duration of motility and fertilizable life-span. The extension of functional life-span is accomplished(3) without metabolic utilization of the added amino acid. Experiments with fowl sperm(4) have also shown an extension of motile life-span as a result of the addition of an amino acid to the saline diluent. It was of interest, then, to learn whether or not such agents would be effective in extending the life-span of mammalian spermatozoa. The experiments with the invertebrates were performed at temperatures in the range of their usual environment. Life-span studies on spermatozoa of mammals have generally been carried out at low temperatures since the purpose evidently is to extend, as much as possible, the period during which the sperm may be stored before use in artificial insemination. It should be noted, however, that mammalian spermatozoa must survive at body temperature in the female genital tract for some time after insemination before the eggs are encountered. Their duration of survival at such temperature may, then, be an important factor in determining whether or not successful fertilization will occur.

In view of these considerations the present experiments with bovine spermatozoa were performed at both body and refrigerator temperatures. The effect of the amino acid solution was compared with that of isotonic NaCl, balanced salt solution and the yolk-citrate diluent(5) which, following the discovery(6) of the efficacy of hen's egg-yolk, is generally employed for storage of bovine spermatozoa at low temperatures.

Material and methods. Semen was obtained from Holstein and Guernsey stud bulls of the Roger Jessup Certified Farm† in Los Angeles, Calif. The samples were gradually cooled and kept at 2° to 4° until use at the laboratory, usually between 1¾ and 6¼ hours later. Sperm counts of the various samples ranged from 1 to 14 x 10^8 per ml. The semen was diluted 1/100 and 1/1000 in the various test solutions. Pyrex, glass-stoppered, 25 ml Erlenmeyer flasks were used with 4 ml of the diluted semen. In the experiments at 38.6°C, these were shaken at 80 c.p.m. and 5 cm excursion. In the experiments at 4°C, 50 ml flasks were used with 4 ml of diluted semen and these were not shaken. Estimates of the percentage of motile sperm were made hourly in the 38.6°C series and daily in the 4°C series, with 2 or 3 readings taken during the first period. Motility was scored as 0, 1, 5,

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TABLE I. Duration of Motility of Bovine Spermatozoa at 38.6°C and 4°C in Glycine as Compared with Balanced Salt and Yolk-Citrate Diluents.

	No. of exp.	Mean half-life (hr)	Mean increase over corresponding control: Hr	Fraction	Mean 90%-life (hr)	Mean increase over corresponding control: Hr	Fraction
38.6°C; 1/100 semen							
Balanced salt	15	5.9			11.2		
.13 M glycine	14	.6	−5.7 ± .9†	−13.9 ± 3.7†	1	−10.3 ± 1.1†	−24.4 ±10 *
.075	15	4.7	−1.2 ± 1.2	−1.9 ± 1.9	12.9	1.7 ± .9	.2 ± .1
.038	15	7.2	1.3 ± .9	1.5 ± 1	12.8	1.6 ± 1	.2 ± .1
.019	15	7.2	1.3 ± .7	.4 ± .3	12.5	1.3 ± .9	.2 ± .1
.009	14	7	.8 ± .7	.1 ± .2	12.2	1 ± .6	.1 ± .1
Yolk-citrate	14	8	2.3 ± .8*	.5 ± .2	10.6	−.7 ± 1.1	.2 ± .3
38.6°C; 1/1000 semen							
Balanced salt	15	3.2			4.6		
.13 M glycine	14	.5	−2.9 ± .5†	−10 ± 5.1	.7	−4 ± .7†	−14.7 ± 6.4*
.075	15	3.8	.6 ± .9	.2 ± 1	7.9	3.3 ± .9†	1 ± .4*
.038	15	6.9	3.7 ± .5†	5.3 ± .3†	9.2	4.6 ± .5†	1.2 ± .2†
.019	15	6.6	3.4 ± .5†	6.5 ± .4†	8.7	4.1 ± .4†	1.3 ± .4†
.009	14	5.7	2.3 ± .5†	3.4 ± .3†	7.7	3.1 ± .6†	.8 ± .2†
Yolk-citrate	14	2.3	−.8 ± .7	−4.9 ± .5†	6.6	2.1 ± 1.2	.5 ± 1.2
4°C; 1/100 semen							
Balanced salt	7	14			33		
.13 M glycine	7	4	−10 ± 2†	−3.9 ± 1.2*	11	−22 ± 10	−9.3 ± 2.1†
.075	7	14	0 ± 4	−.1 ± .4	34	1 ± 7	.1 ± .3
.038	7	15	1 ± 2	.2 ± .2	31	−2 ± 5	0 ± .2
.019	7	15	1 ± 1	.2 ± .2	33	0 ± 4	.1 ± .1
.009	7	14	0 ± 1	0 ± .1	33	0 ± 4	.1 ± .1
Yolk-citrate	7	185	171 ±18†	13.2 ± 2 †	300	267 ± 26†	9.1 ± 1.3†
4°C; 1/1000 semen							
Balanced salt	7	12			23		
.13 M glycine	7	3	−9 ± 1†	−3.5 ± .6†	9	−14 ± 4*	−5.4 ± 1.7*
.075	7	11	−1 ± 2	−.2 ± .3	25	2 ± 2	.1 ± .1
.038	7	14	2 ± 1	.2 ± .1	24	1 ± 1	.1 ± .1
.019	7	13	1 ± 1	.1 ± .1	24	1 ± 1	.1 ± .1
.009	7	14	2 ± 1	.2 ± .1	24	1 ± 1	.1 ± .1
Yolk-citrate	7	47	35 ± 6†	2.9 ± .4†	97	74 ± 17†	3.2 ± .8†

* Exceeds 5% level of significance. † Exceeds 1% level of significance.
See text for further explanation of table.

10, 25, 50, 75 and 100%. In each set of experiments aliquots of a single semen sample were diluted in the various glycine solutions, a balanced salt solution and yolk-citrate. The balanced salt solution was of the following composition: 1000 ml of 0.15 M NaCl, 22 ml 0.15 M KCl, 195 ml 0.10 M $MgCl_2 \cdot 6H_2O$, 103 ml 0.10 M Na_2SO_4, 35 ml 0.10 M $CaCl_2$, 5.9 ml 0.15 M $NaHCO_3$. The pH was adjusted when necessary to 7.3-7.4. The glycine solutions were prepared by dilution of an isotonic stock solution in the balanced salt solution. The yolk-citrate consisted of 1 volume of hen's egg yolk plus 1 volume of 3.6% $Na_3C_6H_5O_7 \cdot 2H_2O$ and contained 0.3% sulfanilamide, 1000 units per ml potassium penicillin G, and 1000 units per ml of dihydro streptomycin sulphate. In certain experiments, yolk-citrate without the antibacterial agents was also employed. Tests were also made of glycine and balanced salt solutions containing the antibacterial agents. No marked effect of the presence or absence of these agents on the duration of motility was observed and the data are not included in the present report(7-11).

Results. Summarized data for 15 sets of experiments at 38.6°C and 7 sets of experiments at 4°C are presented in Table I. Two end points of motile life-span are employed for presentation of the data. These are termed half-life and 90%-life, and represent the time at which half and 90%, respectively, of the initially motile sperm had become immotile. The values in most cases were obtained by interpolation of consecutive readings of percentage motility. Two methods of ex-

pressing the effect of treatment on the duration of motility are employed in the table. One consists simply in the means of the difference, in life-span, between a particular test solution (glycine or yolk-citrate) and the corresponding (*i.e.*, same semen sample) balanced salt solution control. Negative values, of course, signify shorter life-span than in the control. The standard error of the means is given and significance, as determined by the t-value(12,13), is indicated by a single asterisk or a double asterisk where it exceeds the 5% or 1% levels respectively. The other method, listed under the heading "fraction" in the table, gives the means of the ratio of the difference in life-span between test solution and corresponding control to life-span in the control or the test solution, depending upon which is the lower value. Thus, these fractions have negative values when motility in the control solution lasts longer than in the test solution.

With the 1/100 dilution of semen at 38.6°C the data show no significant effect of glycine, in the concentration range 0.009 to 0.075 molar. In 0.13 M glycine a considerable decrease in life-span is shown. In yolk-citrate there is a small increase in half-life span, with significance slightly above the 5% level, when expressed as the difference from the balanced salt control, but no significant effect when expressed as fractional increase. This increase is not obtained for the 90% end point by either method of expression.

With the 1/1000 dilution of semen at 38.6°C the data show highly significant increases in life-span (both the half-life and 90%-life end points) in 0.009, 0.019 and 0.038 molar glycine solutions. Thus, the mean percentage increases in half-life span in these solutions are 340, 650 and 530 respectively. In 0.13 molar glycine there is again a decrease in life-span. In yolk-citrate there is either no significant effect or a decrease, depending upon which end-point and which method of expression is used.

At 4°C, with both dilutions of semen, the yolk-citrate shows the well-established favorable effect on the life-span of the sperm. Glycine solutions of 0.009 to 0.075 molar have no significant effect, while 0.13 molar again gives a decrease which is somewhat less marked than in the 38.6°C series.

Discussion. The results show that the favorable effect of yolk-citrate in extending the life-span of bovine spermatozoa at low temperature in not manifested at 38.6°C. Glycine, which extends considerably the life-span of spermatozoa of invertebrates(1-3) and also of fowl(4), has, at concentrations of 0.009 to 0.038 M, a marked favorable effect on bovine sperm at 38.6°C and 1/1000 dilution of semen. At the 1/100 dilution and this temperature, the effect is not shown. This is consistent with the lesser effect obtained in the experiments with invertebrates and fowl, when higher concentrations of sperm are used. No significant effect of glycine on the life-span of the bovine sperm is found at the lower temperature, except for the injurious effect of a high concentration (0.13 M) shown at both temperatures.

One of the factors that may be involved in the results is the effect of aeration. MacLeod(14) suggested that H_2O_2 was formed by mammalian (human) sperm under aerobic conditions and this shortened their life-span as compared with anaerobic conditions. Addition of catalase and hemoglobin prolonged aerobic survival, presumably by destruction of the H_2O_2 whose formation was attributed(15) to dehydrogenation of succinic acid. Tosic and Walton(16,17) identified as H_2O_2 an inhibitor of respiration and motility formed by bovine spermatozoa incubated aerobically in yolk-phosphate. They found that catalase and peroxidase could reverse the inhibition. The H_2O_2 was shown to be formed in the presence of a dialyzable fraction of egg-yolk and by phenylalanine, tyrosine and tryptophane but not by any of ten other amino acids, including glycine, that were tested. Prince and Almquist(18) showed that agitation of the partially filled tubes in which bovine sperm are customarily stored decreased livability to a statistically significant degree. Ram semen on the other hand seem to require periodic aeration for maintenance of motility according to Gunn(19). Van Demark *et al.*(20) found that the presence of oxygen decreased somewhat the livability and motility of bovine sperm in yolk-citrate at 46.5° and 5°C and

that the deleterious effect could be largely eliminated by addition of catalase. However, in a later investigation(21) no improvement from added catalase was obtained in either livability or fertility. It is suggested(21) that the difference may be correlated with whether or not the semen was diluted before cooling.

From these and other investigations there appears to be some uncertainty as to the effect of aeration on the life-span of mammalian spermatozoa under various conditions. The present experiments at 38.6° were performed under aerobic conditions since *in vivo* the spermatozoa would be under such conditions in the female genital tract. (The O_2 tension within the uterus of the rabbit has been found (22) to be 20-45 mm Hg which is evidently ample for aerobic metabolism of the spermatozoa.) In the 4°C series the low fluid level (ca 2 mm) also insured essentially aerobic conditions even without shaking. As the results show the yolk-citrate at this temperature gave the usual great increase in life-span of the spermatozoa. That the yolk-citrate fails to show such effect, or may even be deleterious at the higher temperature, might possibly be due to more rapid formation of a toxic agent such as H_2O_2, but in the absence of additional information there are many other possible interpretations based on changes in metabolic patterns of cells at different temperature.

The action of glycine was of special interest in this work in view of the beneficial effects of various amino acids shown in the earlier experiments with invertebrate sperm. At relatively low concentrations this amino acid also extends the life-span of the bovine spermatozoa. The effect is manifested only at the higher temperature and with the more dilute semen. At high concentration (0.13 M) the glycine is injurious. According to Tosic and Walton(17) glycine is among the ten amino acids that do not cause H_2O_2 formation. This agent is, then, presumably not responsible for the injurious effect of high concentrations of glycine and, of course, not for the favorable effect of lower concentrations. Possibly the extension of life-span obtained with glycine in bovine spermatozoa involves the same sort of action as in the case of the invertebrate and fowl sperm. In these the experiments(1-4) suggest that the amino acids act by binding certain heavy metal ions normally present in trace amounts in the dilution medium. Further unpublished experiments on invertebrates with other kinds of chelating agents support this view. However, preliminary tests on sperm with the metal-chelating agents diethyldithiocarbamate and ethylenediaminetetraacetate have given no very marked favorable effect. The present evidence does not, then, permit a decision to be made on the extent to which the presence of trace metals in the dilution medium may be involved in the life-span of bovine sperm, nor on the manner in which glycine is able to extend the life of these cells.

Summary. The addition of glycine, at concentrations of 0.009 to 0.038 molar, to a balanced salt diluent, increases considerably the motile life-span of bovine sperm at 38.6°C. The effect is manifest in 1/1000, but not in 1/100, dilutions of semen. It is not exhibited at 4°C. In yolk-citrate the usual extension of life-span is obtained at 4°C, but at 38.6°C there is either no significant effect or a decrease as compared with the balanced salt solution controls. The results are discussed in relation to experiments on sperm of invertebrates and fowl in which the life-span extending action of amino acids is attributable to the binding of trace metals present in the dilution medium.

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Thermal Inactivation of a New Vibrio Phage.*† (19879)

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Susceptibility of bacteriophages to inactivation by heat has been observed by several workers to be a general phenomenon(1-4a). Nanavutty(5) studied rates of inactivation of a coli-dysentery phage exposed to various temperatures. He plotted the logarithms of residual active phage against time and obtained concave curves. He also observed a difference in heat susceptibility of the phage when different suspending fluids were used. Application of an accurate analytical method for phage assay to experiments on the thermolability of staphylococcal phage demonstrated that: 1) heat inactivation at various temperatures follows the course of a monomolecular reaction; and 2) the temperature coefficient is of a magnitude characteristic of protein denaturation(6). Other races of bacteriophage have since been shown to behave similarly during heat inactivation(7,8).

Recently we have been making a survey of the properties of a new Vibrio-phage system. The present papers deal with some interesting aspects of the heat inactivation of this particular phage.

Materials and methods. The virus-host system under study was recently isolated from San Francisco Bay mud. The bacterium is of the genus Vibrio, and will not grow in ordinary media without the addition of NaCl. A concentration of 3% sodium chloride supports maximum growth of the organism and optimal phage production. The phage lysate was prepared in yeast extract mineral 3% salt medium‡ and was passed through a Seitz filter (Type ST) to secure a bacteria-free suspension of phage at pH 7.9. When it was desired to compare the susceptibility of the phage suspended in various fluids, the lysate was ultracentrifuged, the supernatant fluid decanted, and the phage resuspended in the medium indicated, *i.e.*, M/15 phosphate buffer pH 7.9, M/15 phosphate buffer pH 7.9 plus 3% NaCl, 1% aqueous yeast extract plus NaCl in concentrations of 5, 4, 3, 2, 1, 0.5, 0.25, and 0.125%, respectively and no NaCl. The phage was exposed to a given temperature by dispensing 0.5 ml aliquots into prewarmed test-tubes and immediately placing these in the water bath at the desired temperature. At the end of each time interval a sample was removed from the water bath and immediately

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‡ The composition of Yeast Extract Mineral 3% salt medium is:

Yeast Extract (Difco)	%	Bacto dehydrated	1%
			%
$MgSO_4$	.05	NaCl	3.0
K_2HPO_4	.1	Tap water	10
NH_4NO_3	.1	Dist. water q.s.	90

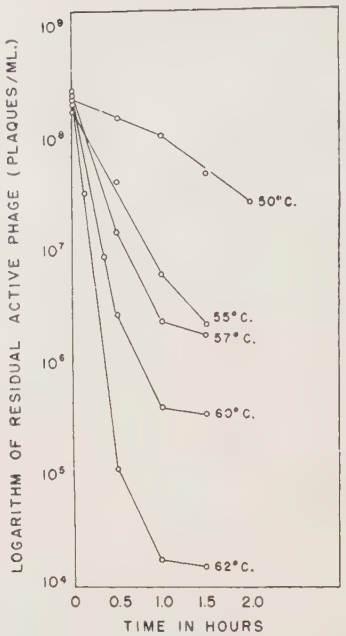

FIG. 1. Heat inactivation of Vibrio phage in yeast extract mineral 3% NaCl medium.

diluted in nutrient broth containing 3% NaCl. The residual active phage was determined by plaque count according to a modification of the original method of Gratia(9). The procedure employed, *i.e.*, using separate small aliquots of the phage suspension for each experiment, has several technical advantages; it is open to the objection that the results may not apply to a single large volume. We have checked this point by conducting parallel experiments with both methods and have obtained identical results.

Results. When phage lysates were exposed to various temperatures, and the logarithm of the numbers of remaining phage particles plotted against time, it was observed that the rate of inactivation did not remain constant. Inactivation at 50°C became faster after one hour; at 55°C the plot most nearly followed a straight line, but at higher temperatures, 57°C, 60°C, 62°C, there was a break in the curve at 0.5 hour and the rate decreased after this time (Fig. 1).

For comparison of the behavior of the phage in various environments it was resuspended in each of the following fluids, M/15 phosphate buffer pH 7.9, M/15 phosphate buffer pH 7.9 plus 3% NaCl, and 1% aqueous yeast extract. Susceptibility to heat inactivation was found to be different in each case. However, the same tendency to produce concave curves with rising temperatures was revealed (Fig. 2 and 3).

Discussion. From the data presented in Fig. 1, 2, and 3, it is possible to reach certain conclusions: 1) Susceptibility of the Vibrio phage to inactivation by heat is influenced by the nature of the suspending fluid. Of those used in this work M/15 phosphate buffer at pH 7.9 rendered the phage most vulnerable. The addition of 3% NaCl to the buffer increased the resistance. Likewise phage in 1% yeast extract was more resistant than in the buffer alone. However, phage activity was most resistant to high temperatures when the filtered lysate (containing both yeast extract and 3% NaCl) was exposed. This finding is in accord with the reported properties of other bacteriophages. The influence of suspending fluid on susceptibility to inactivation by heat has been demonstrated for a coli-dysentery phage by Nanavutty(5), for

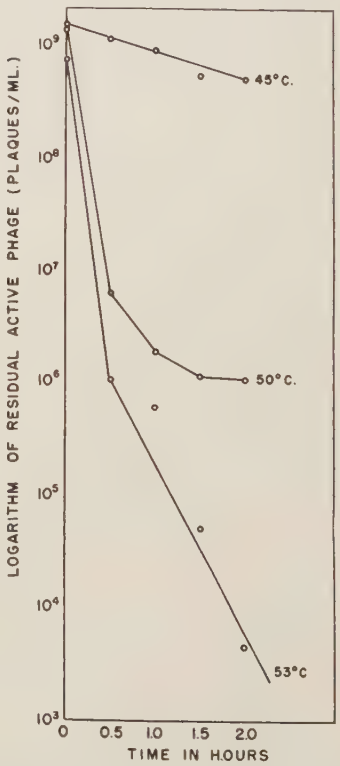

FIG. 2. Heat inactivation of Vibrio phage suspended in M/15 phosphate buffer pH 7.9.

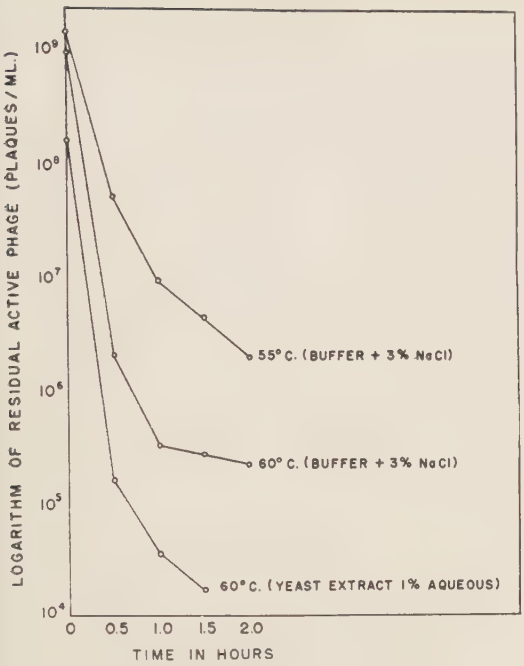

FIG. 3. Heat inactivation of Vibrio phage suspended in M/15 phosphate buffer pH 7.9 + 3% NaCl and in 1% aqueous yeast extract.

coli and staphylococcus phages by Burnet and McKie(10), for a megatherium phage by Gratia(11), and for the group of "T" coli phages by Adams(8). 2) Changes in the rate of phage inactivation with time occur in all of the suspending fluids. This indicates that the shape of the curves is not due to either the presence of a high concentration of electrolytes or the yeast extract alone. 3) Approaching the highest temperatures at which the inactivation can be studied, marked decreases in the rate occur after 90% of the phage activity has been lost. 4) In general, if essential experimental conditions are met, the curves for heat inactivation might be expected to be straight line plots of the sort reported by Krueger for staphylococcal phage(6). We have considered several possible explanations of the deviations noted above. It is conceivable for example that we are dealing with two races of phage which are characterized by different degrees of thermoresistance. If this were the case exposure to 60°C for 2 hours should completely destroy the more thermolabile race and should leave only the thermoresistant one. Such an experiment was performed and numerous plaques were picked from the remaining active phage. New phage preparations were developed separately on suspensions of the host cell and filtered lysates were made. The thermal inactivation characteristics of these lysates were determined and it was found that they behaved exactly as did the original phage from which they had been derived, *i.e.*, the experiment gave no indication that the observed changes in rate of thermal inactivation were due to separate races of phage. 5) Since reactivation of thermally inactivated phage has been reported by Krueger and Mundell(12) one might explain the present data in such terms if conditions were such that reversal of denaturation occurred only after 0.5 hour. However, experiments described in the accompanying paper on thermal shock(13) seem to preclude this possibility. 6) The presence of a hypothetical protective substance developing after the first 0.5 hour of heating could account for the reduced rate of inactivation. To investigate this possibility lysate was heated at 60°C for 0.5 hour, diluted with warmed (60°C) broth containing 3% NaCl and exposure to 60°C was continued for 1.5 hours longer. Theoretically, dilution of the postulated protective substance should diminish its effect on phage and inactivation should continue at the original rate. Actually no such result was obtained; the curve for inactivation corrected for dilution was the same as that of the undiluted control. 7) A skewed curve of distribution of thermoresistance among the phage particles composing the original lysate could account for the sets of curves in Fig. 1, 2, and 3, without involving any genetic factor. We have no evidence disproving the existence of such distribution.

Conclusions. 1. Susceptibility of the Vibrio phage to thermal inactivation is influenced by the nature of the suspending fluid. Decrease in rate of inactivation with time occurs in all of the fluids tested. 2. Several possible explanations for deviation of the inactivation curves from the plots predicted by the Mass Law have been considered.

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Comparison of Creatinine and Inulin Clearance in the Dog During Hypoxia.* (19880)

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The creatinine/inulin clearance ratio has been reported by Shannon to be 0.99 in unanesthetized dogs(1,2). This agreement was found to be maintained during anesthesia (chloralose), but discrepancies began to appear in the isolated perfused dog kidney probably subjected to varying degrees of anoxia at low urine volumes where the ratio averaged 0.86(3). The desire to gain assurance that the creatinine clearance remained an index of glomerular filtration rate during hypoxic conditions in the dog prompted the present investigation in which this clearance was compared to the accepted standard of reference, the inulin clearance.

Methods. Pentobarbitalized animals were used in these experiments. Arterial blood pressure was recorded with a mercury manometer by direct cannulation of a femoral artery, and the trachea was cannulated for connection to a Douglas bag. Urine was collected by direct catheterization of the ureters. Substances to be analyzed for clearance were infused at a constant rate by a motor driven syringe. A moderate diuresis was instituted by infusion of Ringer's solution prior and during the experiment. *Hypoxia* was produced by inhalation of an 8% oxygen, 92% nitrogen mixture. In animals which tolerated the procedure well, alternate stages of low oxygen mixture and room air were observed, with a pair of urine collection periods of ten minutes duration at each stage, for a total of five stages (Table I). In some animals difficulties were encountered as the result of continued breathing of the low oxygen mixture (fall in blood pressure, respiratory paralysis) so that experiments were of briefer duration. Although inhalation of 8% oxygen produced the lowest level of blood oxygen content compatible with survival, in two experiments the kidney was subjected to an even greater degree of hypoxia by a technic of perfusion of the kidney *in situ* with venous blood while the animal breathed 8% oxygen. This was done with the aid of a pump previously described (4), which took blood from the right ventricle through a cannula introduced via the external jugular vein and delivered it into the renal artery at pressure kept at arterial pressure levels by proper adjustment of the stroke volume of the pump. An experiment of this type appears in Table II. *Blood oxygen* content was determined by the method of Van Slyke and Neill(5) in samples taken mid-way in each stage of the experiment. Since these were single determinations they cannot be taken as average values for oxygen content, but serve only as a relative index of the degree

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TABLE I. A Representative Experiment Showing Effects of Inhalation of 8% Oxygen-92% Nitrogen Mixture on the Creatinine and Inulin Clearance. (20 kg female. Values are for one kidney only.)

Period	Blood O_2,* vol/%	MABP,† mm Hg	Urine vol, ml/min	Creatinine P, mg/%	Creatinine U, mg/%	Creatinine UV/P, ml/min	Inulin P, mg/%	Inulin U, mg/%	Inulin UV/P, ml/min	C_{CR}/C_{IN}
A. Room air										
1	14.1	122	1.30	11.3	240	27.6	23.3	500	27.9	.99
2		121	1.60	12.6	210	26.8	23.6	397	27	.99
B. 8% oxygen										
3	9.6	122	1.60	14	233	26.8	23.6	394	26.7	1
4		121	1.42	13.8	268	27.6	23	457	28.2	.98
C. Room air										
5	13.9	123	1.44	14.8	296	28.9	25	467	26.9	1.07
6		121	1.42	15.3	307	28.4	26.6	475	25.4	1.12
D. 8% oxygen										
7	10.4	120	1.44	16.5	305	26.6	27.4	469	24.6	1.08
8		115	1.25	17	364	26.8	27.1	543	25	1.07
E. Room air										
9	14.7	121	1.27	17.8	374	26.8	27.6	542	24.9	1.07
10		125	1.33	18.3	369	26.8	28	529	25.1	1.06

* Blood O_2 content is given as that combined with hemoglobin. The rather low values noted during periods in which room air was inhaled are attributed to the effects of anesthesia on respiration and to reduction of red cell hematocrit (to 37%) resulting from hemodilution by Ringer's infusion.

† Mean arterial blood pressure was typically briefly elevated immediately after instituting 8% oxygen inhalation. It tended to subside by the time urine collection was started.

TABLE II. A Representative Experiment in Which Perfusion of the Kidney with Venous Blood Was Carried on While Breathing 8% Oxygen. (13.2 kg male. Values for one kidney only.)

Period	Blood O_2, vol/%	MABP, mm Hg	Urine vol, ml/min	Creatinine P, mg/%	Creatinine U, mg/%	Creatinine UV/P, ml/min	Inulin P, mg/%	Inulin U, mg/%	Inulin UV/P, ml/min	C_{CR}/C_{IN}
A. Room air										
1	14.1	129	2.15	11.6	124	23	19.2	229	25.6	.90
2		132	2.27	11.7	131	25.5	19.9	221	25.3	1.01
B. 8% oxygen										
3	5.5	134	1.32	11.9	212	23.5	20.4	350	22.7	1.03
4		135	1.05	12.2	281	24.3	19.7	485	25.9	.94
C. 8% oxygen + venous perfusion										
5	2.1	133	1.32	13.2	158	15.8	20.5	278	17.9	.88
6		134	1.60	14.1	94	10.6	21.7	176	12.9	.82
D. Room air										
7	14.6	133	4.10	16	89	22.8	24.4	141	23.7	.96
8		134	4.13	16.7	93	23	24	140	24.1	.95

of saturation. Carotid artery blood was usually analyzed, but when the pump was used the sample was drawn from a stopcock in the renal perfusion circuit, supplied either by carotid or venous blood. *Chemical methods* for the clearance determinations employed the alkaline picrate method for determination of creatinine in urine and sodium tungstate filtrates of the plasma. The modified Seliwanoff reaction of Higashi and Peters(6) was employed for the inulin determination.

Results. Results were obtained from six animals. The creatinine/inulin clearance ratio averaged 1.01 (S.D., ± 0.066) in 28 clearance periods in which the animals were breathing room air. This compares with an average of 0.97 (S.D., ± 0.06) found by Higashi and Peters(6) employing the same inulin method, and a ratio of 0.99 (S.D., ± 0.048) found by Shannon using another inulin method(2).

During hypoxia produced by inhalation of 8% oxygen this correspondence was well maintained, as shown in the representative

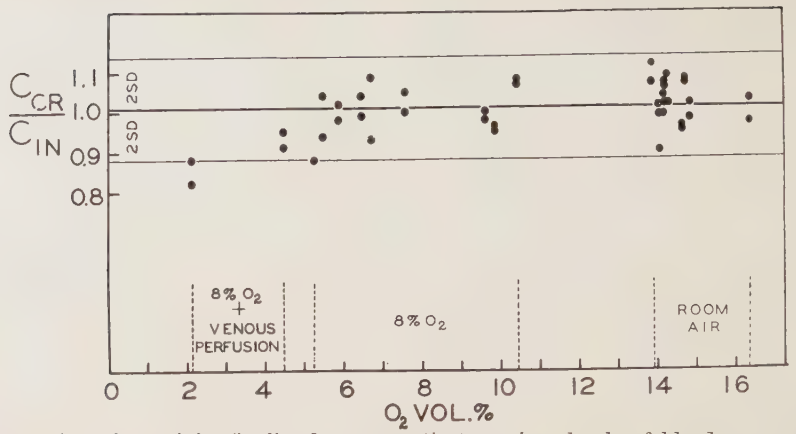

FIG. 1. Relation of creatinine/inulin clearance ratio to various levels of blood oxygen content. The mean ratio and ± 2 S.D. are based on a total of 28 clearance observations while breathing room air. During 8 clearance periods blood O_2 content was not measured; hence only 20 control values appear in the figure.

experiment. A total of 17 observations fell within the limits of ±2 S.D. from the mean of the control in a range of oxygen content of 5.5 to 10.4 vol. %.

In two experiments in which perfusion of the kidney with venous blood was superimposed on a generalized hypoxia created by inhalation of 8% oxygen, a slight reduction in the ratio appeared. This can be noted in Table II. In these experiments there was a tendency for the absolute clearance values to be reduced. At the low value of 2.1 vol. % the ratio declined to 0.82, the lowest observed ratio in these experiments. However, in the other experiment with oxygen content of 4.46 vol. % ratios were 0.91 and 0.95.

Discussion. The present investigation gives assurance that the clearance of creatinine can be used as a valid index of glomerular filtration rate during hypoxia with oxygen content of the blood as low as 5.5 vol. % continued for periods as long as 30 minutes. Below this level of oxygen, the tendency was noted for the creatinine clearance to be diminished relative to the inulin clearance. This may have been the result of back diffusion of creatinine through the tubular epithelium by mechanisms discussed by Shannon(3). The possibility is not excluded that both substances may back-diffuse during the most severe hypoxic states, with creatinine diffusing more rapidly than inulin. However, this was not believed to be the case in the present experiments in which 8% oxygen was inhaled because the absolute clearance values were well maintained, as illustrated in the experiments of Tables I and II.

Summary. The creatinine/inulin clearance ratio remains close to unity during hypoxia in anesthetized dogs with blood oxygen content as low as 5.5 vol. % resulting from inhalation of an 8% oxygen mixture. Supported by the fact that absolute clearance values do not decrease, it is concluded that the creatinine clearance remains an adequate measure of glomerular filtration rate under these conditions.

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Effect of Deficiency of Magnesium and Other Minerals on Protein Synthesis. (19881)

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A recent study(1) stressed the importance of potassium in protein synthesis. We undertook to determine the importance of magnesium and other mineral elements in this process.

Method. Female albino rats (Wistar) of approximately 200 g were subjected to protein depletion, as in the work of Cannon *et al.*(1), by means of a ration deficient only in protein (2% of total calories, with casein as sole protein source) for 4 weeks. During this period an average of 25% of their initial weight was lost. The animals were then divided into 4 groups of 4 animals each (approximately the same number as used by Cannon) except Group 4, which consisted of 3 animals. All were given a basal ration containing, per 100 g, 9 g casein which had been subjected to hot alcohol extraction (Vitamin-Free Test Casein, from General Biochemicals, Inc.) 75 g cane sugar, 7 g butter, 1 g U.S.P. cod liver oil, 5 g non-nutritive cellulose fiber (General Biochemicals, Inc.) and 3 g aqueous mixture containing 300 mg choline (as the monobasic citrate), 1 mg thiamine hydrochloride, 4 mg niacin, 2 mg riboflavin, 2 mg calcium pantothenate, and 1 mg pyridoxine hydrochloride. The water soluble vitamin content† of this ration was similar to that of Cannon *et al.* (1). The importance of using hot alcohol-extracted casein in a magnesium-deficient ration was originally stressed by Kruse *et al.*(2). Group 1, serving as a control, received a complete mineral supplement, which had the same total of each mineral element as the Hawk-Oser modification of the Osborne-Mendel salt mixture(1). Four g of this mineral supplement was added to each 100 g of basal ration fed to Group 1. Group 2 received an amount of mineral supplement equivalent to that of Group 1 but lacking magnesium; K_2SO_4 and Na_2CO_3 were substituted respectively for $MgSO_4$ and $MgCO_3$ to give the same amounts of SO_4 and CO_3, thus making the total Na and K slightly higher in the salt mixture of Group 2 than of Group 1. The mineral supplement of Group 3 consisted solely of NaCl and KCl (thus lacking Mg, Ca, P, Fe, and trace elements), the total Na and K being equal to these totals in the supplement fed Group 1. Group 4 received no mineral supplement.

Results. (See Table I) Group 1 (complete supplement) had to be restricted in its consumption of the basal ration to keep the intake close to the amount consumed by Group 2 (Mg deficient). Groups 2, 3 and 4 were permitted to eat *ad libitum.* Group 3 (NaCl and KCl) ate less than Group 2 (Mg deficient). On a per rat basis, Group 4 (no salts) ate almost as much as Group 3. Table I shows the total basal ration consumed per rat for each group during the first 10 days on this ration and the mean weight gain per rat for each group (with the standard deviation of the mean) during this period. Group 1 gained the most weight per rat, and each succeeding group gained less than the preceding one on a per rat basis. The differences in mean weight gain per rat between succeeding groups were statistically significant, p being less than 0.01 for the differences between succeeding groups in all instances.

Comments and conclusions. Group 1 (complete supplement) gained over 40% more than Group 2 (Mg-deficient), although Group 1 ate only 4% more than Group 2. Cannon *et al.* (1) found that deficiency of potassium resulted in a decreased rate of protein repletion. Although muscle tissue has less than one-tenth as much magnesium as potassium, deficiency of magnesium was as profoundly deleterious to protein regeneration in these experiments

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† Kindly contributed by Dr. Louis Freedman of U. S. Vitamin Corp., New York.

TABLE I. Effect of Different Mineral Supplements on Weight Gain.

Group	Mineral supplement	No. of rats	Avg consumption of basal ration per rat (g/10 days)	Avg wt gain per rat (g/10 days)
1	Complete	4	112	30.5 ± 2.33
2	Mg-deficient	4	108	21 ± 1.47
3	NaCl and KCl	4	83	15.5 ± 1.04
4	No salts	3	81	11.3 ± .88

as was lack of potassium in the experiments of Cannon *et al.*(1) where the food intakes were kept approximately equal. Group 2 showed no outward anatomical or physiological signs of magnesium deficiency(2) other than decrease in appetite and in weight gain.

Group 2 (Mg-deficient), which ate 30% more than Group 3 (NaCl and KCl), gained over one-third more than the latter, whose salt supplement (NaCl and KCl) resembled that frequently given when protein hydrolysates or amino acids are administered parenterally. If Group 3 (deficient in Mg, Ca, P, Fe, and trace elements) had been forced to eat as much as Group 2 (Mg-deficient), the weight gain per rat of Group 3 would hardly be expected to have exceeded that of Group 2. That is, a complete mineral supplement might here be expected to promote at least 40% more protein regeneration than would a supplement of only KCl and NaCl at the same level of protein and caloric intake.

The food consumption per rat of Group 3 (NaCl and KCl) was only 2% greater than for Group 4 (no salts), but the weight gain per rat of Group 3 was over one-third greater than for Group 4. Hence the supplement of NaCl and KCl apparently exerted its favorable influence on protein regeneration without significantly stimulating appetite.

In these conclusions, as in those of Cannon *et al.*(1), it has been assumed that gain in body weight is essentially a gain in muscle and visceral tissue of normal protein content. However, it should be borne in mind that water storage can affect weight. Thus Group 4 (no mineral supplement) may have gained less weight than Group 3 (NaCl and KCl) partly because Na and Cl are needed for a normal increment of extracellular fluid while K would play a similar role for intracellular fluid. Neither nitrogen balance nor fat deposition was investigated. The effect of the various mineral deficiencies on the absorption of protein from the gut also was not studied in this investigation.

Summary. Controls receiving a complete mineral supplement gained over one-third more weight than rats on a magnesium deficient diet. These, in turn, gained over one-third more than animals receiving only KCl and NaCl as their mineral supplement, while the latter gained about a third more than rats not receiving mineral supplement. The unfavorable effect of magnesium deficiency on the synthesis of protein appears to be approximately as great as that reported for potassium deficiency, although there is far more potassium than magnesium in muscle and viscera.

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Some Biological Characteristics of Holothurin.* (19882)

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Late in 1951, while working at the Lerner Marine Laboratory on Bimini, Bahamas, B. W. I., the second-named author undertook to survey a number of marine organisms with the view of determining the general toxicity as well as any possible tumor-inhibiting potency of aqueous extracts derived from their tissues. To determine their systemic toxicity the extracts of macerated whole bodies were assayed in terms of the minimum amount, parenterally injected, required to kill a 20 g white Swiss mouse. Among representatives of some 50 different marine genera so sampled was the sea cucumber, *Actinopya agassizi;* a crude water extract made from the whole body was found to be lethal to mice in very small amounts, but the exact minimum lethal dose was not determined at the time, nor was any effort made to identify the tissue responsible for the toxic factor. Working independently in 1952, and also at the Lerner Marine Laboratory, the first-named author observed that a desiccated preparation of the Organ of Cuvier of the sea cucumber contained a factor which was highly toxic against mice, certain amphibia, and fish. Nigrelli designated this water soluble factor as *holothurin,* and has described its preparation and its general characteristics(1).

The present report deals with the toxicity of this material to several protozoa grown in synthetic liquid culture, and to suspensions of tumor cells.

Effect on protozoa. A water solution of the desiccated holothurin material shows no loss in toxic potency when autoclaved for 30 minutes at 120°C. It may therefore be added directly to the nutrient medium of a microbiological assay organism before sterilization and inoculation. Using methods described by Hutner and coworkers(2), the holothurin material was assayed in various minute concentrations for its effect on the growth of three different protozoa: *Tetrahymena pyriformis, Euglena gracilis,* and the freshwater chrysomonad *Ochromonas malhamensis.* The results of these tests are presented in Table I. It is seen that 2.2 mg of the holothurin material per 100 ml of nutrient liquid is sufficient to stop all growth of *Tetrahymena* and *Ochromonas. Euglena* tolerates a somewhat higher concentration, but it too is ultimately poisoned to complete inhibition by the factor.

TABLE I. Effect of Holothurin on Growth of Various Assay Microorganisms.

Level of holothurin in medium*	Growth†			
	Ochromonas, pH 5.2	Euglena, pH 3.5	Euglena, pH 6.5	Tetrahymena, pH 6.6
—	6	2.1	.90	4+
.0001	7	2.7	—	4+
.0003	6	2	.90	4+
.001	5.6	2	.95	4+
.003	7.3	2	1	3+
.01	8.6	2	.90	3+
.03	7.5	2.2	.90	+
.1	.0	2.2	.86	0
.3	.0	1.8	.22	0
1	.0	.52	.22	0
3	.0	.30	—	0

* Level—ml of a 22 mg/ml water solution of holothurin, made up to 100 ml of nutrient medium.

† Growth—after 6 days incubation at 25°C, read either in photodensity units or (for *Tetrahymena*) estimated. +=trace growth; 4+=profuse growth.

Effect on tumor cells. Using methods described earlier(3), uniform suspensions (ten million tumor cells per ml) were prepared from 7-day-old implants of mouse Sarcoma 180. Two ml volumes of such a suspension were pipetted into 15 ml tapered centrifuge tubes. Amounts, ranging from 1μg to 1 mg, were added to the 2 ml cell suspensions. The tubes were rotated in a roller apparatus (4) at 34°C for one hour, at the end of which time 0.2 ml volumes (each containing 2 x 10^6 tumor cells) were drawn out of the tubes and

* We wish to express our thanks to Mr. Jack Storm for having carried on technical aspects of the microbiological work herein described, and to Dr. S. H. Hutner with whom Mr. Storm worked. We are grateful to Dr. C. M. Breder, Jr., who generously made the facilities of the Lerner Marine Laboratory available to us in connection with this problem.

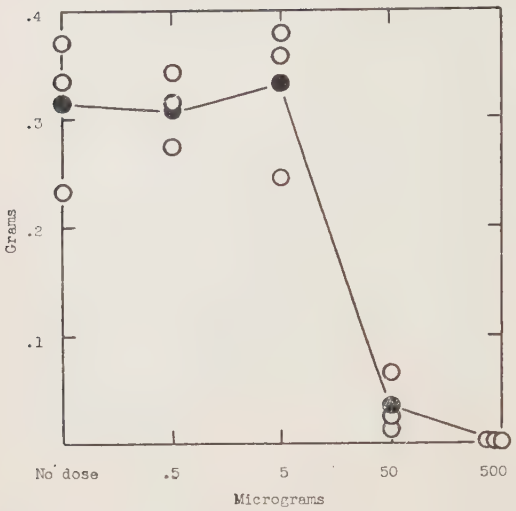

FIG. 1. Growth of tumor cell inocula pre-treated in various concentrations of holothurin. Horizontal axis indicates concentrations of holothurin-containing preparation per ml of tumor cell suspension; vertical axis indicates wet wt of tumors at the end of 7 days. Each open point on the graph represents the mean of one 5-animal experiment; solid points represent means of the three experiments. Scatter of individual wt determinations omitted because it was not dissimilar to that described in(3).

inoculated subcutaneously into mice in the usual site for axillary implantation.

At the end of a 7-day period, the growths resulting from the cell inocula were dissected out and wet-weighed. Five mice were used for each assay, and each experiment was repeated 3 times. White Swiss male mice were used throughout the experiment.

Fig. 1 indicates the effect of this treatment on the growth potency of the cell suspensions.

Comment. From a comparison of the data of Table I and Fig. 1, it is seen that the effect of holothurin, both on protozoa and on suspended tumor cells, is one with an abrupt threshold. The dosage difference between "no effect" and "complete killing" is very small. The significance of this is as yet not known, although the presence in holothurin of multiple toxic factors may be indicated. The toxicity of holothurin to protozoa in synthetic media is lessened by the addition of complex natural materials, such as mixtures of nucleic acids and protein hydrolysates(5). Identification of the effective antagonists in these mixtures may provide a clue to the mechanism of holothurin toxicity.

Inasmuch as very few thermostable natural organic materials have been described which are highly toxic to animals and also to protozoa and suspended tumor cells, further investigation of the active toxic principle of the sea cucumber would seem to be warranted.

Summary. *Holothurin*, a thermostable toxic factor derived from the Organ of Cuvier of the Caribbean sea cucumber, acts as a powerful poison to several protozoa grown in pure culture. The same material applied *in vitro* to cells of mouse Sarcoma 180 markedly reduces their growth potency.

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Release of Enzyme from Human Leukocytes on Damage by Bacterial Derivatives.* (19883)

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Bacterial polysaccharides are well known for their pyrogenic effect. The need for knowledge concerning their effect on cellular metabolism is apparent. Detailed studies on the alteration in the metabolic processes of human leukocytes on exposure of the cells to these substances have been carried out in this laboratory by Martin, McKinney, Chaudhuri, and Green(1). Such studies are time-consuming and require large quantities of cells for their completion. A screening method has been reported elsewhere(2) whereby less detailed information concerning adverse metabolic effects can be obtained more rapidly and with relatively small quantities of leukocytes. The method depends on the release from injured cells of cellular contents; the released substance measured is an enzyme with the properties of lysozyme. The data obtained by this method have correlated well with those obtained by the more detailed methods of metabolic study. Although little information is derived by the screening method as to the nature of the adverse metabolic effects, the simplicity of the method makes possible a wider range of comparative observations. The present study compares the injurious effect of a variety of bacterial polysaccharides and other bacterial derivatives on human leukocytes. The relative effect of the substances varies from individual to individual donor. The possible significance of this variation is discussed.

Materials and methods. Subjects. Patients with no evidence of organic disease or with non-infectious diseases excluding primary metabolic or hematologic disorders. All subjects were only mildly ill, clinically. *Leukocyte suspension.* Sedimented leukocytes prepared by a method described previously(2) were resuspended in a buffered balanced physiologic salt solution plus 186 mg % dextrose to a final count of 5500-6500 leukocytes per cmm. *Bacterial derivatives.* PV and PS Piromen; concentrates of *E. coli* and *B. subtilis;* glucosamine (Baxter Laboratories, Inc.). Friedlander B polysaccharide (Dr. Harold Ginsberg). Pneumococcus type III SSS (Dr. O. T. Avery). Shwartzman-potent meningococcal filtrate (Dr. B. S. Black-Schaffer). Staphylococcus toxin (Lederle Laboratories). Tuberculin PPD (Dr. Florence Siebert). Appropriate dilutions were made in physiologic unbuffered saline for the experiments tabulated in I and in balanced salt solution plus glucose for II, to the final concentrations noted in the tables. *Lysozyme substrate.* Bacto Lysozyme Substrate (Difco Laboratories, Inc.) stock suspension was prepared and diluted for use by a method previously described. *Experimental procedure.* 0.7 ml of the diluent or of the test substance (in concentration 5 times the desired final concentration) was added to 2.8 ml of leukocyte suspension in 25 ml sterile siliconed flasks. The mixtures were incubated at 37°C in a Warburg shaker for 1 hour before use. In each instance, 1.0 ml of the test suspension was added to 1.5 ml of lysozyme substrate, and readings were obtained at zero time and at 20 minutes at 540 mμ in a Coleman Junior Spectrophotometer, as previously described (2), 100% transmission being set by use of a blank tube containing 1.0 ml of the test suspension and 1.5 ml of buffer. All tests were run in duplicate. Percent lysis of the substrate was calculated by the formula 100 minus (the optical density at 20 minutes divided by the optical density at 0, times 100). A correction was then made for the initial speed of the enzymatic reaction, since the significance of differences between control and test observations is considerably altered by

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TABLE I. Effect of Varying Concentrations of Bacterial Polysaccharides on Human Leucocytes as Indicated by Enzyme Release from the Cell.

Leucocytes from various human subjects, plus:	Index of injury of leucocytes by added bacterial polysaccharides* —Conc. of polysaccharide added/ml— 10 γ	5 γ	.5 γ	.05 γ
PS Piromen		155	111	71
PV "		108	76	66
PV "		136	98	95
E. coli concentrate		188	102	77
B. subtilis "		135	73	43
Friedlander B. polysaccharide		64	59	55
Pneumococcus III SSS	70	61	11	

* See text for derivation of index which relates each test reading to the control. The larger the index, the greater the injurious effect on the cells as compared to control leucocytes. An index of ± 24 or more is significantly altered (calculated using 2 S.D. of the method).

TABLE II. Comparison of Indices of Injury* to Human Leucocytes from 4 Subjects on Exposure of the Cells to Various Bacterial Polysaccharides.

Bacterial derivative added:		Human leucocytes from subject No. 1	No. 2	No. 3	No. 4
PS Piromen	4 γ/ml	137	107	99	69
PV "		205	172	143	123
E. coli concentrate		107	94	92	81
B. subtilis "		137	84	130	83
Friedlander B polysaccharide		77	56	53	60
Pneumococcus III SSS		83	19	83	67
Glucosamine		−11	4	3	−17
Staph. toxin	1:25	67	136	119	96
Meningococcal filtrate	1×10^{-5}	152	83	83	84
PPD	.02 mg/ml	77	11	69	36

* See footnote, Table I.

this factor(2); the following index was determined: (% lysis in the presence of leukocytes exposed to test substance) minus (% lysis in the presence of control leukocytes), divided by (% lysis in the presence of control leukocytes), times 100. The larger the index, the greater the lysis of substrate in the presence of test leukocytes as compared to control leukocytes. Control experiments on the method, using 2 S.D. in the calculations, indicate that an index of ±24 is significantly altered.

Results. The design and results of experiments are summarized in tabular form. Table I indicates the rough quantitative difference in injurious effect with varying concentrations of bacterial polysaccharide. The leukocytes used in each polysaccharide series were obtained from a different subject.

Table II tabulates comparative data for all the bacterial derivatives studied, each of the 4 series of 10 derivatives being run on a leukocyte suspension from a single subject.

Discussion. The injurious effect of various bacterial derivatives on human leukocytes is demonstrated by a method which depends upon the release of cellular contents when the cell is injured. The method is sensitive and may find useful application in the detection of pyrogen-contamination in solutions which are not in themselves injurious to leukocytes. Rough quantitation of the amount of pyrogen present in such solutions would be possible only if the pyrogen concentration were low, the effect of the test solution being compared with the effect of known pyrogen concentrations on the same leukocyte suspension. Determination of pyrogen levels in plasma or serum has not seemed feasible because of the presence in the test samples of significant amounts of the lysozyme-like enzyme measured.

When leukocytes from a single individual are exposed to the entire series of bacterial derivatives used in the present study, it is noted that the injurious effect of PV Piromen is relatively large and that the effects of

Friedlander B and of Pneumococcus III SSS are relatively small. Of further interest is the variability from individual to individual on comparison of the effect of the various bacterial derivatives on their leukocytes. It seems reasonable to postulate that this may reflect the previous experience of the donor individual with the various bacteria concerned. Detailed studies are in progress to test this hypothesis.

Summary. By a method which detects cell injury by measurement of a lysozyme-like enzyme released from the cell, the adverse effect of various bacterial derivatives on human leukocytes is demonstrated. Variation in degree of effect from individual to individual is evident.

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Erythrocyte Potassium Levels in Pernicious Anemia and Non-Tropical Sprue. (19884)

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Taylor and coworkers(1) have shown that compounds known to inhibit cholinesterase activity interfere with the maintenance of the *in vitro* potassium concentration gradient of erythrocytes in normal human subjects. In these systems, the amount of inhibitor needed to effect the gradient was more than enough to bring cholinesterase activity to zero. In pernicious anemia, this enzyme activity is depressed during relapse, elevated during the period of reticulocytosis, and normal during remission(2). Although red cell cholinesterase never reaches zero in pernicious anemia in relapse, the degree of variation seen in the course of this disease made it of interest to determine whether erythrocyte potassium levels were abnormal in relapse, and whether any significant change took place during the period of reticulocytosis.

Methods. This study included one patient with non-tropical sprue and 5 persons with typical Addisonian pernicious anemia in relapse. Each case in the latter group showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, and megaloblastic bone marrow. Roentgen examinations of the gastrointestinal tract, urinalyses and blood chemistry determinations disclosed no abnormalities. All of the patients were treated with intramuscular injections of liver extract. The patient with non-tropical sprue showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, megaloblastic bone marrow and flat oral glucose tolerance curve. There were frequent liquid stools containing abundant fatty acid crystals. Roentgen evidence of spasm, segmentation and "puddling" of the barium column in the small intestines were reported. He was treated with citrovorum factor,* intravenously. The results of this study will be presented elsewhere(3). In all of the patients hemoglobin, red blood cell, hematocrit, plasma and whole blood potassium determinations were made three times a week, leukocyte counts once a week, and reticulocyte estimations daily. Potassium analyses were made with a flame photometer with lithium as an internal standard. Erythrocyte potassium values per liter were calculated by subtracting plasma potassium from whole blood values using the hematocrit, and correcting by a factor of 0.95 for trapped plasma.

Results. All of the patients showed satis-

* Citrovorum factor supplied by Dr. J. M. Ruegsegger, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE I. Potassium Levels in a Patient with Non-Tropical Sprue (A.N.) and 4 Patients with Pernicious Anemia from Relapse to Early Remission.

Name	RBC, $\times 10^6$	Retic., %	K meq/ l RBC	Day of treatment
A.N.	1.69	.5	97	1
	1.92	14.4	104.5	6
	2.62	4.2	95	10
	3.09	1.2	103	17
F.C.	1.30	1.3	99	1
	1.15	2.1	97	2
	1.60	17.8	93	5
	2.11	7.8	96	10
	2.47	2.2	106	13
	2.85	1.3	106	16
T.W.	1.85	.8	99	1
	1.58	1.8	97	4
	1.95	16.6	96.5	7
	2.42	5.1	97.5	12
	2.62	2.7	99	15
C.S.	2.63	1.5	91.5	1
	2.94	1.4	102	3
	2.63	4.8	97.5	7
	2.81	5.8	104	10
	3.15	3.2	103.5	15
	3.40	1	100	21
G.K.	1.82	.6	80.5	1
	2.16	18.6	89.5	8
	2.97	8.3	90.5	12
	3.19	2.2	84.5	16
	3.32	1.8	93	22
A.S.	2.82	1.1	87	1
	2.96	2.4	92	4
	2.95	2.1	97	7
	3.21	3.7	90	11
	3.81	2.2	88	16

factory hematologic and clinical remissions. Reticulocyte responses were in the optimal ranges and regeneration of red blood cells took place rapidly. Table I summarizes the pertinent data and shows that no significant change in erythrocyte potassium level was noted in any stage of red blood cell production. Patient G. K. showed a slightly lower level throughout the study than did the others. This may represent a depletion of total body storage of potassium as suggested by Hutt (4). Values for the other patients fell within the normal values suggested by other investigators(5). In view of these negative results experiments in K^{42} turnover under similar conditions appear warranted.

Summary. Erythrocyte potassium content is unaltered in the relapsed stage of pernicious anemia and non-tropical sprue. No change is noted during the period of reticulocytosis and red blood cell regeneration.

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Atheromatous Changes in Aorta, Carotid and Coronary Arteries of Choline-Deficient Rats.* (19885)

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Evidence suggesting an association of dietary deficiency in rats with the occurrence of vascular damage has not, to our knowledge, been previously reported.† We have recently observed in our laboratory pathological changes, which resemble in some respects those of atheroma, in the major arterial trunks (aortas and carotid arteries) and in the coronary arteries of rats maintained up to 216 days on diets low in choline. A preliminary account of the extent and nature of these lesions is embodied in this paper.

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† We have been informed personally (April, 1952) that in the chronic choline deficiency studies by Salmon, Copeland, and Engel, Agri. Exp. Station, Alabama Polytechnic Institute, lesions similar to those described in this paper have been observed in the aorta and other blood vessels of rats over a period of several years.

Experimental procedure. Male rats of the Wistar strain reared in our own colony were used for this investigation. Since young rats may die from acute renal damage due to choline deficiency(1), animals with initial weights between 180 and 200 g were used in order to reduce the number of deaths from this cause. The rats were housed in individual all-metal cages with a false bottom of coarse wire screen. One group of animals was fed *ad lib* a severely hypolipotropic *diet A*, of the following percentage composition: peanut meal (solvent process) 6; alpha soya protein (Glidden's) 6; hydrogenated vegetable fat (Primex) 20; salts 3; vitamin mixture 1; cellu flour 2; sucrose 61.8; cystine 0.15; alpha tocopheryl acetate 0.035 and cod liver oil concentrate 0.015. The other group of rats was given *diet B* in which the protein moiety was slightly increased at the expense of the sucrose (peanut meal 8, alpha soya protein 8), a mixture of animal and vegetable fat (beef fat 15, corn oil 5) replaced the hydrogenated vegetable fat in diet A and the alpha tocopheryl acetate was increased to 0.100%; the other ingredients were the same as those in diet A. The preparation of the diets, ingredients of the salt mixture and of the vitamin mixture and details concerning the care of the animals have been published previously(2). At the end of 3 to 4 months, food consumption had markedly decreased and a considerable loss in body weight had occurred. Some of the rats died, and examination of the livers revealed the beginning of cirrhosis. In an attempt to prevent further deaths among the animals consuming diet A, a supplement of 2% casein, 1% fibrin and small amounts of choline chloride (0.04 to 0.02%) were included for about two weeks. During this short period, stimulation of appetite promoted gains in body weight of from 30 to 40 g, associated with a marked improvement in their general appearance. Choline chloride was removed from the diet and the protein supplement was continued for a third week. The animals were then transferred to the original hypolipotropic diet A until they were sacrificed. Rats fed diet B ingested the unsupplemented diet B throughout the experiment. One hundred and sixteen animals were studied. Ninety-one received diet A and 49 of these were subjected to both gross and microscopic examination. The remaining 42 were dissected but microsections were not taken, because of extensive post mortem changes in most instances. Twenty-five rats received diet B. Of these, 11 were examined both grossly and microscopically and 14 were autopsied but microsections were not taken, in most cases again because of post mortem changes. All animals in which vascular lesions were grossly apparent were subjected to microscopic examination even in the presence of post mortem changes.

Histological examination included preparation of paraffin sections of all thoracic and abdominal viscera, and of the aorta. Frozen sections of the aorta and of the hearts of 29 animals fed diet A and of 12 fed diet B were stained by Wilson's technic(3). Frozen sections of the hearts of all of the 61 rats subjected to extensive histological examinations were not prepared as in the initial stages of the study it was not appreciated that lipid deposits might be found in the coronary arteries. Paraffin sections were routinely stained with haematoxylin and eosin. A variety of special tinctorial procedures was applied to certain sections including methods for the demonstration of elastic tissue, ceroid (Oil Red O stains of paraffin sections), connective tissue, hemosiderin pigment, and special cytological technics for the demonstration of the cells of the islets of Langerhans and of the anterior lobe of the pituitary. Estimations of liver lipids and of serum cholesterol were carried out in some cases. The biochemical data will be published elsewhere.

Results. Aortas of 9 of the 91 rats fed diet A and of one of the 25 rats fed diet B were grossly thickened, tortuous and studded with calcified plaques (Fig. 1, 2). Definite pathological changes were found in microsections of aortas of an additional 16 rats (14 fed diet A; 2 fed diet B). In 8 of these, the lesions were early or minimal in nature (6 fed diet A; 2 fed diet B). Thus, in a total of 26 animals, some degree of vascular damage was encountered. Lesions of the coronary arteries were found in frozen sections of 8 of the 41 hearts so examined. The results and

FIG. 1. Gross appearance of thoracic aorta lesions graded as 3+ of a rat fed diet A. The vessel is increased in diameter and a number of plaques and rings (white) are visible along its entire length. × 4½.

FIG. 2. Gross appearance of aorta of a rat (fed diet A) showing lesions graded as 4+.

Aortic arch is at the left and a small portion of the upper abdominal aorta is at right. Annular, calcified bands occur at fairly regular intervals in the thickened wall along the entire length of the vessel shown. The rings are largest and heaviest in the thoracic portion and the arch; they lie opposite the intervertebral junctions, as indicated by the position of the ribs. ×3.

FIG. 3. Microscopic appearance of a frozen section stained for fat (Wilson's technic) of the normal aorta of a rat. Note absence of subintimal tissue. The intima (top) illustrates the *upper limit* of cellularity in control rats. At this magnification, the typical normal rat aorta would exhibit only one or two endothelial cells with scantier cytoplasm than those shown. ×800. (Compare with Fig. 5.)

FIG. 4. The avascularity of the media of the normal aorta of a rat injected intrav. with india ink is demonstrated by this preparation which has been cleared *in toto* with benzyl benzoate and photographed without sectioning. The intimal surface (left) overlies the transparent media (pale grey) which is free of injection media. The adventitia (right) appears almost jet black for it is supplied by a dense network of capillaries which have been filled with ink. The adventitial capillary plexus terminates sharply at the junction between adventitia and media. × 24.

FIG. 5. Preparation and magnification as for Fig. 3 illustrating one of the earliest stages (1+) of the aortic lesion encountered in a choline-deficient (diet A) rat. The intima is hyperplastic and the cells are filled with stainable fat droplets (black) which are so numerous that the nuclei (approximately 10 in the field shown) are almost obscured. In the mid-zonal portion of the underlying media can be seen a focus of early degeneration and prenecrosis (dark grey).

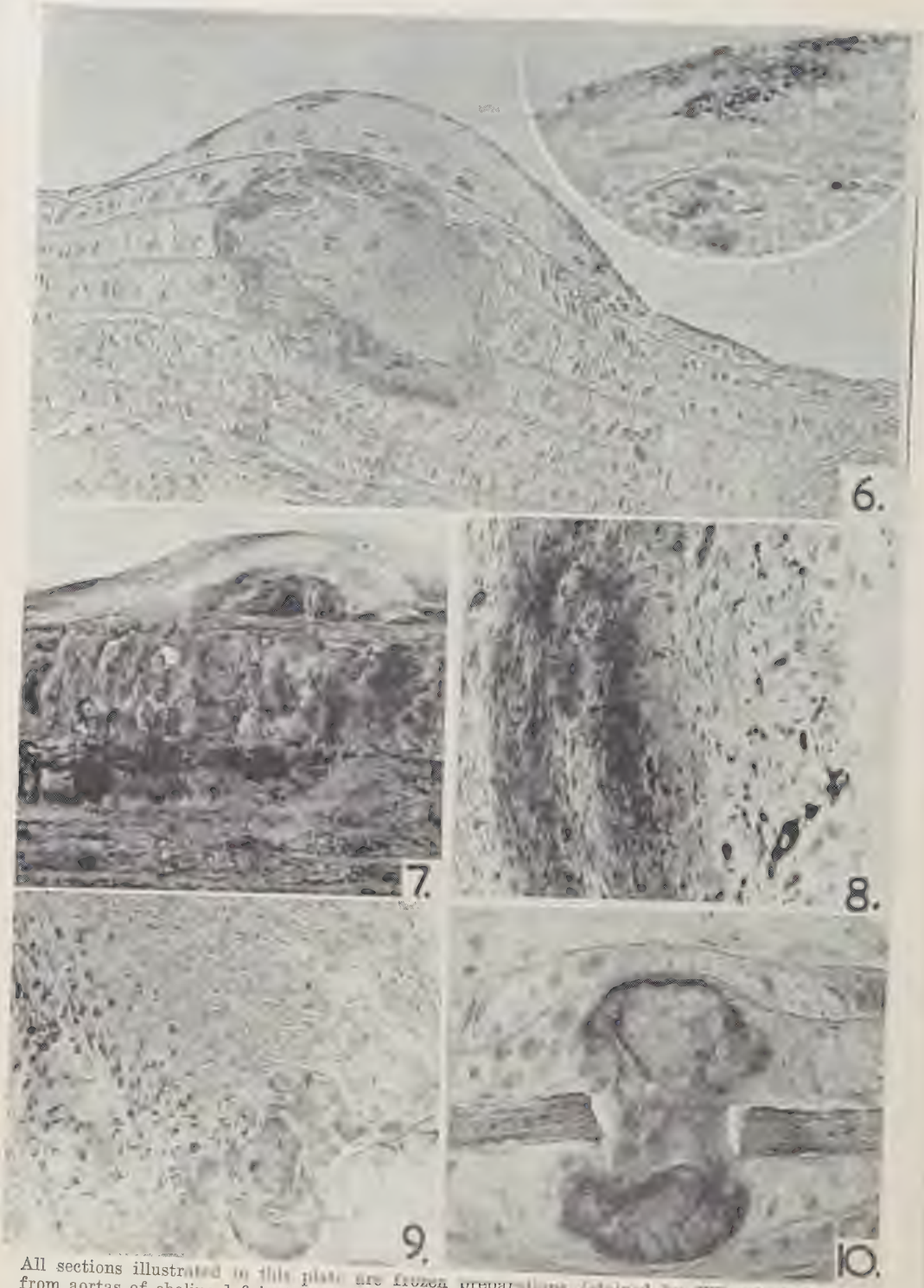
some associated findings are summarized in Table I. The gross and microscopic appearance of the aortas and other vessels are described below. In several animals, blood pressures were measured (direct method) and all were found to fall within normal limits, thus confirming earlier studies of the effect of subjecting rats to continuous and prolonged dietary choline deficiency(4).

The lesions on gross examination (Fig. 1, 2).

TABLE I. Summary of Pathological Findings in 26 Rats Fed Low-Choline Diets, in Which Lesions of the Aorta or Coronary Arteries Were Present.

Rat No.	Diet	Vascular lesions		Liver		Kidney damage	Time on diet, days
		Aorta	Heart	Fat	Cirrhosis		
5	A	4+	+	1+	3+	3+	210
31	A	4+	?	1+	4+	2+	151
85	A	4+	0	3+	3+	3+	140
106	B	4+	0	2+	3+	2+	170
923	A	4+	?	2+	4+	3+	201
979	A	4+	+	2+	2+	1+	173
859	A	3+	+	2+	3+	2+	219
924	A	3+	+	1+	4+	3+	200
940	A	3+	?	1+	4+	2+	176
988	A	3+	?	3+	3+	1+	167
12	A	2+	?	2+	3+	1+	188
15	A	2+	?	1+	3+	2+	192
16	A	2+	?	2+	3+	2+	195
46	A	2+	?	2+	3+	1+	167
48	A	2+	0	1+	4+	2+	169
92	A	1+	2+	2+	3+	2+	184
934	A	2+	0	2+	2+	1+	195
938	A	2+	+	1+	3+	2+	195
91	A	1+	+	2+	3+	2+	189
110	B	1+	0	2+	2+	1+	170
116	B	1+	0	2+	2+	1+	169
801	A	1+	0	2+	2+	1+	205
869	A	1+	0	2+	3+	2+	216
882	A	1+	+	1+	4+	2+	214
902	A	1+	0	1+	4+	2+	209
997	A	1+	?	1+	2+	2+	180

Grossly visible changes are designated as either 3+ or 4+. Changes indicated by + or 2+ were evident only microscopically. This grading of lesions (+ to 4+) is purely arbitrary and bears no relation to similar classifications that may have been employed by others in studying lesions in other species including man. In those instances where a ? is found, microscopic examination was not carried out for this particular feature (see text).



All sections illustrated in this plate are frozen preparations (stained by Wilson's technic) from aortas of choline-deficient rats fed diet A, with the exception of Fig. 9 which shows an aorta from an animal fed diet B.

FIG. 6. Later stage of an aortic lesion, than that shown in Fig. 5, of an intimal plaque

graded as 2+. The plaque is composed of hypertrophied and hyperplastic endothelial cells, many of which contain stainable fat droplets (black). The subjacent media has undergone more extensive necrosis than that shown in Fig. 5, and small amounts of calcium salts have been deposited in the necrotic tissue. × 250. The inset (upper right, × 800) shows a few of the intimal cells under higher magnification to demonstrate the fat droplets more clearly.

FIG. 7. A single, hypertrophied intimal cell from an early stage of a typical aortic lesion (1+) contains stainable fat droplets in the cytoplasm just at the left of the enlarged nucleus. The cell is swollen and has an hyaline, 'glassy' appearance. The midzonal portion of the media (extreme bottom of field) exhibits degenerative changes indicating early necrosis. × 800.

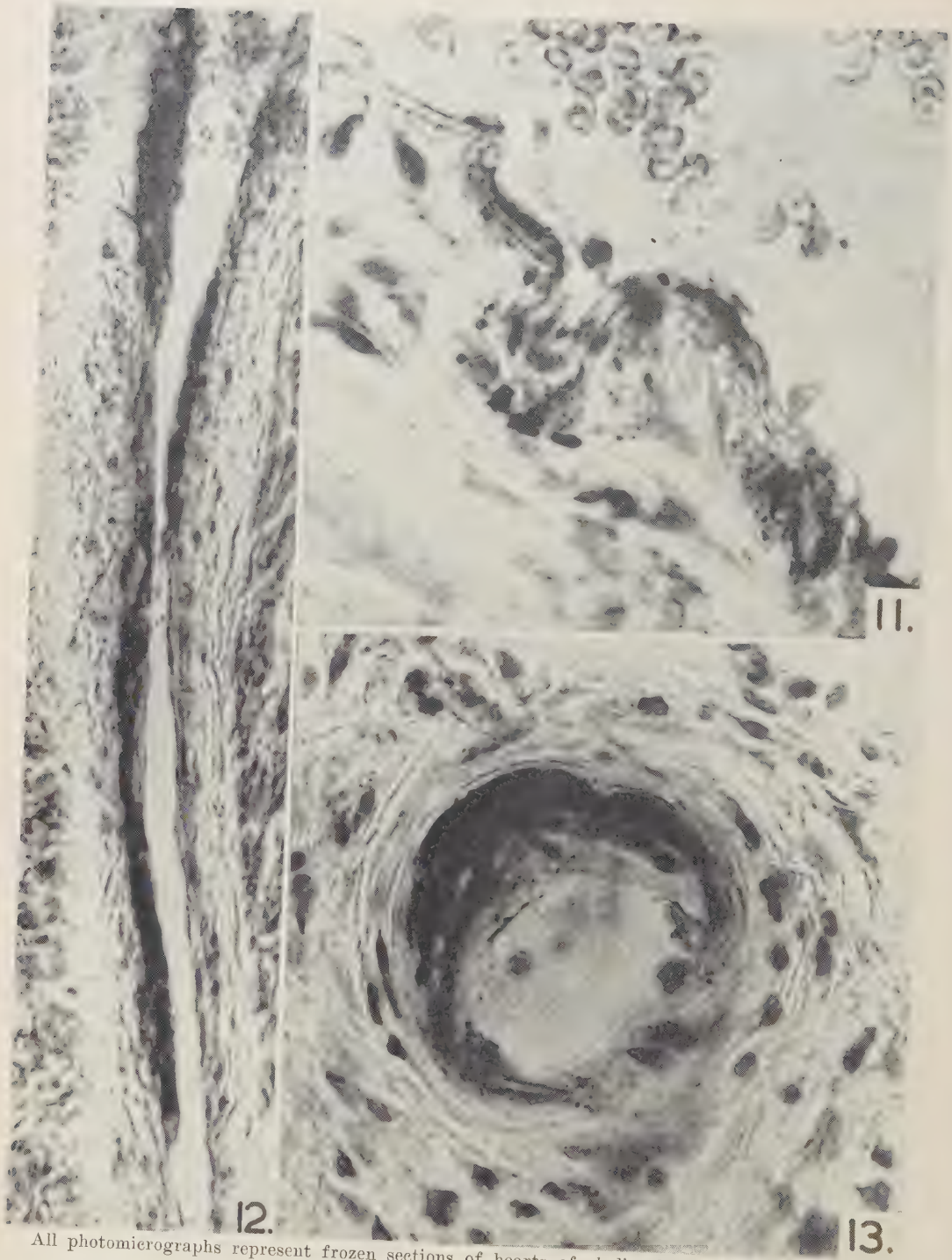
FIG. 8. Appreciable amounts of stainable lipid (black), some of which reacted positively to histochemical tests for cholesterol, are present in the media of this aorta with lesions graded as 3+. The intima is greatly thickened (taking up about one-third of the field at the right) and contains stainable fat (black) within hyperplastic endothelial cells and some macrophages. Intimal lipid was always negative to histochemical tests for cholesterol. × 100.

FIG. 9. A grossly thickened aorta (graded as 4+) is shown in cross section with intima in extreme upper right and a small portion of adventitia at extreme lower left. Calcium deposits appear dark grey; stainable fat, black. The junction between intima and media could not be identified. Note fibrosis. × 100.

FIG. 10. Typical callus ("collar-button lesion") has formed around the site of fracture of the media which has been replaced throughout its mid-zone by a longitudinal bar of calcium salts. The lesions in this aorta were graded 4+. A portion of the greatly thickened intima is included in the upper portion of the field. × 100.

In the most advanced cases (classified as either 4+ or 3+, Table I), the aortas were increased in diameter and frequently deviated a few millimeters to one or the other side of the vertebral column. Scattered throughout could be seen grey to yellow plaques varying from 2 to 4 mm in their greatest dimensions. Sometimes these were in the shape of elongated or oval patches which lacked any apparent constant orientation to the vessel wall (Fig. 1), but most frequently they formed annular bands which often completely encircled the vessel (Fig. 2). Sometimes these bands were most prominent opposite the intervertebral discs and, if numerous, gave the aorta an appearance resembling that of the trachea. Lesions were always most abundant and advanced in any one animal, in the ascending portion and the arch of the thoracic aorta, and decreased in number and size farther away from the heart. The bases of the common carotid arteries at their sites of origin from the aortic arch were affected in a few rats in which the lesions were present in severe degree (4+). In two rats (No. 5 and 859), small yellow plaques were evident at the root of the aorta, and were situated in the wall of the aortic sinus where they partially surrounded the openings of the coronary arteries. They were the only lesions associated with coronary vessels that were detected on gross examination. On palpation, the largest lesions were stony hard and were nearly always grey in color, in contrast to smaller, softer plaques which were frequently quite yellow when examined with the aid of a hand-lens. At the site of the largest plaques, the outer surface (adventitia) of the vessel was elevated, but this was not the case with smaller ones which were most easily visualized from the inner (intimal) surface. Even in the earliest cases, which could be observed grossly, the intimal surface was elevated over the site of a plaque.

Microscopic examination Fig. 3-13. *Normal aorta of the Wistar rat.* Certain characteristics of the rat's aorta are worthy of mention, because they differ from those of the human aorta. The intima of the rat consists of a single row of endothelial cells (Fig. 3) directly overlying the subjacent elasto-muscular media. The nuclei of the intimal lining have not been observed to number more than four per high power field (42X objective with 10X ocular). Areolar connective tissue between the intima and media is lacking in the aorta of the rat, so that there is virtually an absence of subintimal tissue. By injecting India ink into the animal's vascular system (by a technic described previously)(5), it was demonstrated that *vasa vasorum* do not penetrate the media of the aorta (Fig. 4). A fine meshwork of capillaries exists in the adventitial tissue but terminates at the medial-adventitial junction. It would appear then that the nutrition of the media must be wholly dependent on the dispersion of fluid from the lumen of the rat aorta outwards and from the capillaries of the adventitial vascular network



All photomicrographs represent frozen sections of hearts of choline-deficient rats which had been fed diet A. Wilson's stain.

FIG. 11. Stainable fat droplets (black) may be seen on and within the intima of this coronary artery illustrated in longitudinal section. The lumen containing red blood cells is in the upper right half. The media appears normal. × 800.

FIG. 12. Stainable fat (black) fills the thickened intima and a portion of the underlying

media for a considerable distance along the wall of this coronary artery which has been sectioned longitudinally. There is no medial necrosis. × 300.

FIG. 13. Crescentic hypertrophy and hyperplasia has developed in both the intima and media of this coronary artery cut in cross-section. The intima is loaded with masses of stainable fat (black). Note the "onion-skin" layering of the media. × 800.

inwards. The central zone of the media, midway between these two sources, would probably be most susceptible to any interference with the mechanisms responsible for diffusion of fluids into the media.

Initial stages of the aortic lesions. The earliest lesions detected microscopically have been found sometimes in aortas that had appeared normal on gross examination (one-plus, Table I). An increase in the number of endothelial nuclei of the intimal lining had occurred so that the intima appears thickened (Fig. 5). The cells of the hyperplastic intima possess less cytoplasm than normally, but the most striking qualitative change may be observed in the frozen sections stained for fat. In these sections, the hyperplastic intimal cells contain many fine sudanophilic droplets in their cytoplasm. Histochemical tests indicate that these were probably neutral fat, because tests for fatty acids and cholesterol were negative. In some instances the underlying media appears normal, but more frequently there has been interference with the usual intense tinctorial affinity of this tissue for eosin, or for Light Green, indicating early degenerative changes of a prenecrotic nature (Fig. 5). These initial foci of degeneration in the media are always located midway between its inner and outer limits.

Later stages of the aortic lesions. In the less extensive of those lesions which were visible on gross examination (3+), proliferation of intimal endothelium had produced small raised plaques formed by layers of two to five cells (Fig. 6). The amount of stainable fat within these cells had not increased appreciably over that found in earlier lesions. Cells comprising these plaques are often swollen and large, and their cytoplasm possesses a smooth, glassy appearance suggesting a type of hyaline degeneration (Fig. 7). The underlying media in the mid-zone shows varying stages of necrosis and, in a few instances, deposition of small amounts of calcium salts. Fatty change in the media is present in some cases (Fig. 8) and histochemical tests for cholesterol were positive. Cholesterol was not demonstrated, however, in the intima in any of the sections tested.

Advanced stages of the aortic lesions. In the most advanced lesions, in addition to hypertrophy and hyperplasia of the intima, there has been subintimal formation of fibrous tissue in which macrophages containing lipid may be found (Fig. 9). The underlying media is completely necrotic in its mid-zone and is replaced by a bar of calcium salts. In some instances this medial bar had fractured and callus had formed with typical granulation tissue (Fig. 10).

Lesions of the coronary arteries. The earliest change that could be noted consists microscopically of the deposition of stainable lipid droplets *on* and within the cytoplasm of the endothelial lining cells of the largest branches of the coronary arteries (Fig. 11). Sometimes this change has been accompanied by hypertrophy and hyperplasia of the affected cells, but not always. In some cases, for considerable distances the intima and a portion of the underlying media of these vessels are bright red with fat (in frozen sections stained with Oil Red O) which renders the arteries very prominent under the lower magnifications of the microscope (Fig. 12). In more advanced stages of coronary arterial change, the entire intima is swollen with stainable fat and there is evidence that the underlying media has become thickened, for these vessels possess an "onion skin" appearance (Fig. 13). Medial necrosis was not observed in even the most severely affected coronary arteries.

Discussion. The question of spontaneous lesions. It is considered unlikely that the lesions which have been described, were spontaneous in nature. Vascular lesions of this type have not been observed in our colony of rats, including some which were not sacrificed until they had attained an age of 750 days on a stock diet. Spontaneous lesions of both aortas and coronary vessels in Wistar rats

have been reported by others(6,7), but these animals (all more than 600 days old) were twice as old as the choline-deficient rats, and the lesions were not as advanced. None of the animals having arterial lesions associated with dietary deficiency was more than 300 days old.

Dietary factors. Pathological changes in the aorta and coronary vessels were observed only in rats fed the choline-deficient rations. Control animals which had been fed the same basal diets supplemented with choline were not included in these experiments, but in other investigations conducted in this laboratory in which such control groups were studied, evidence of vascular changes has never been noted. It is true, however, that in some of our previous experiments in which similar rats were maintained on less severely hypolipotropic diets for a year or even longer, lesions of the aortas were not observed(2). If present, these changes were so minimal that they escaped notice at routine autopsy. It is possible that secondary factors, in addition to choline deficiency, may have induced, or at least intensified, the vascular lesions so that they were readily detected on gross examination. Such factors might include the nature of the dietary fat (hydrogenated vegetable oil) or the temporary periods of choline supplementation in the early stages of the experiment. It should be noted however that neither of these factors could account for lesions in 3 of the 25 rats fed diet B. The protein content of both diets A and B was extremely low (9 and 12 per cent, respectively) and this should be taken into account when assaying probable factors. Experiments have been planned to investigate these possibilities.

Nature of the lesions and their pathogenesis. Although changes in the coronary arteries of the rats, consisting of intimal lipid deposits and endothelial proliferation, are morphological counterparts of early stages of atheroma in man, this resemblance is not so apparent in the case of the lesions in the aortas of the rats. The latter more closely resemble Mönckeberg's sclerosis of femoral and radial arteries of man, for medial calcification is the cardinal feature of this condition and of the affected aortas of the rats. It is significant in this connection that the aortas of rats resemble these distributing arteries of man more closely than the human aorta. *Vasa vasorum* are limited to the adventitia only in the first two instances, whereas the *vasa vasorum* of the aorta in man penetrate the media(8).

The initial stage of the lesions in the rats' aortas consists of the deposition of intimal lipid. At this period in the development of the disease the microscopic appearance resembles that recently observed by Wilsler *et al.*(9) in the aortas of rats fed diets high in fat and treated with nephrotoxic antiserum, or desoxycorticosterone acetate with and without substitution of 1% NaCl for their drinking water. Bragden and Boyle(10) have described the deposition of sudanophilic material in the intima of rats 24 hours after they had been injected with low-density lipo-proteins obtained from the serum of cholesterol-fed rabbits. The microscopic changes in these rats again resemble the initial changes found in the choline-deficient animals. This type of stainable intimal lipid is reminiscent of that found by Wilens(11) in the artificial production of intimal lipid deposits in excised arteries of man by filtration of normal human blood serum through their walls at normal arterial pressures for 24 hours or longer.

Our studies thus far have suggested that at some stage in the development of the lesions of the liver, kidney and other organs of the choline-deficient rats, factors have operated to favor the deposition of stainable lipid in those portions of the animals' arterial trees where the blood is under highest pressure. These sites were near the heart in the thoracic portions of the aortas, the roots of the common carotids and the stem branches of the coronary arterial trees. Evidence has already been published(2,12) that a state of chronic fat embolism may exist in choline-deficient rats; perhaps it may be a potent factor in the production of vascular changes. In the rat, deposition of intimal lipid in the aorta may be the principal factor directly responsible for medial necrosis. Intimal deposits of lipid may form enough of a barrier that permeation of the mid-zone of the media by nutrient fluids is inhibited to such a degree that at this site the tissue dies and becomes calcified. In man,

intimal lipid in the aorta might not be expected to produce the same result, because medial nutrition is not dependent on fluid permeation alone since *vasa vasorum* penetrate the media. It is hoped that future investigation may elucidate the roles of these factors.

Although there may be some justification for considering that the lesions which have been observed in choline-deficient rats may be related to human atheroma, further discussion is not warranted until the results of more extensive investigations are at hand. It should be of interest however to those concerned with the problem of cardiovascular disease that lesions closely related morphologically to human atheroma can be produced by a dietary deficiency in an animal, the rat, which has previously been considered refractory to aortic disease. It is possible that by following this type of investigation, knowledge of atheromatous conditions in man can be extended.

Summary. Lesions have been observed in the aortas, carotid and coronary arteries in 26 of 116 rats fed a low choline diet for periods up to 216 days. None of these animals exceeded 300 days of age at the time of sacrifice. The initial lesion consists microscopically of the deposition of stainable lipid in the endothelial cells of the intima. In later stages, a proliferation of intimal cells had taken place so that small plaques resulted. In the large vessels (aortas and carotids) the subjacent media had undergone necrosis and eventual calcification. The possible relation of these lesions to atheroma in man is discussed.

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Use of Normal Rabbit Serum in Production of Hypercholesteremia in Cholesterol Fed Rats.* (19886)

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Recently we have found(1) that the hypercholesteremia following the injection of Triton WR 1339, is due to an alteration in the plasma protein complex induced by the injection of this surface active substance. The hypercholesteremia in the rat following the administration of sodium cholate also has been found (2) by us to be due to an *initial* alteration in the plasma protein complex brought about in some way by cholate. In other words, the injection of either of these substances apparently allows the blood of the treated animal to retain a greater fraction of that cholesterol *normally entering and eventually leaving the blood stream.*

These findings suggested the possibility that other forms of hypercholesteremia also might

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TABLE I. Response of Rats Plasmapheresed with Normal Rabbit Serum to High Cholesterol Feeding.

No. of rats	Avg wt (g)	Estimated rabbit serum given (cc)*	Plasma cholesterol (mg/100 cc) Before plasmapheresis	After plasmapheresis Immed.	24 hr	48 hr	144 hr
		A. Rats given rabbit serum—high cholesterol diet					
9	261 (215-300)†	5.5 (4.1-6.5)	45 (31-53)	50 (43-58)	77 (61-93)	115 (92-136)	122 (112-131)
		B. Rats given rabbit serum—cholesterol free diet					
5	258 (214-289)	5.3 (5 -5.5)	44 (40-48)	51 (41-57)	63 (50-69)	68 (60-79)	52 (46-60)
		C. Rats—high cholesterol diet					
5	266 (254-280)	0	49 (43-54)	—	59 (48-82)	64 (43-77)	61 (43-72)

* Calculated as ½ the total vol of rabbit serum given in first plasmapheresis plus total vol given in second plasmapheresis.

† Range.

be due to this mechanism rather than to some intrinsic derangement in the handling of cholesterol by any particular organ or tissue of the body. It therefore was decided to investigate the plasma mechanism underlying the hypercholesteremia occurring in the rabbit placed upon a high cholesterol diet. If the hypercholesteremia of the cholesterol fed rabbit is due *primarily* to some property of its plasma, then transfer of this plasma to a species that ordinarily is refractory to diet induced hypercholesteremia should produce hypercholesteremia in this species when combined with a high cholesterol intake. The following preliminary report indicates that when the rat is so treated, it becomes hypercholesteremic.

Methods. Normal male, Long Evans rats (approximately 12 weeks old) weighing 214-300 g were used in this study. Fourteen of these rats were anesthetized, their blood heparinized and then by means of cannulation of the inferior vena cava, they were bled as completely as possible. Usually 8.0 cc of blood could be obtained from each rat. The blood was centrifuged, the plasma discarded and replaced by an equal volume (usually 3-4 cc) of previously pooled, *normal* rabbit serum (cholesterol content: 62 mg per 100 cc). The reconstituted blood was returned to the rat and after 3 minutes, the entire procedure was repeated. Since the total plasma volume of rats of this weight was approximately 7.5 cc(3), the plasmapheresis probably replaced 66-80% of the rat's own plasma with rabbit serum. No ill effects were observed in the rats following this procedure nor 48 hours later when each rat was injected again with 3 cc of the same pooled rabbit serum. No studies were done to determine how rapidly this serum was destroyed or utilized in the rat but no more than 6% of the initial amount of serum protein was excreted in the urine during the first 24 hours. Following the plasmapheresis, 9 of the 14 rats were given 100 mg of cholesterol in 3 cc of olive oil by stomach intubation and placed thereafter upon a high cholesterol diet. This diet was a mixture of pulverized Purina laboratory chow containing an added 2% cholesterol and 5% corn oil. The remaining 5 plasmapheresed rats were placed upon a cholesterol free diet(4). Five normal, untreated rats also were placed for control purposes upon the high cholesterol diet. Blood samples were obtained before and immediately after plasmapheresis and also, in most of the rats, at the end of 24, 48 and 144 hours at which time the experiment was discontinued. These samples were analyzed for cholesterol according to a previously described method(4).

Results. As Table I indicates, rats whose own plasma had been replaced for a short time at least by *normal* rabbit serum, became hypercholesteremic when placed upon a high cholesterol diet. Their average plasma cholesterol rose from 50 to 115 mg per 100 cc (an increase of 130%) in 48 hours. The

plasma cholesterol rose even higher (122 mg per 100 cc) in those rats tested at the end of 144 hours. This increase did not appear to be due to some *non-specific* reaction of the rat to the introduction of rabbit serum because the five rats given the same serum and in equal amount but placed upon a cholesterol free diet did not have a marked rise in their plasma cholesterol either at 24, 48 or 144 hours. Likewise, the normal, untreated rats placed upon the high cholesterol diet showed only a slight rise in plasma cholesterol.

Discussion. The induction of hypercholesteremia in the rat by substituting rabbit serum for its own plasma and then feeding a high cholesterol diet of course suggests that the hypercholesteremia induced by dietary means in the rabbit itself is also mediated in part at least by a peculiarity of this animal's plasma—a peculiarity which can be transferred to another species. In other words, the plasma phenomenon in respect to cholesterol induced in other animals by Triton or cholate administration may already be an intrinsic property of rabbit plasma. If this is so, the dietary induced hypercholesteremia occurring in the rabbit is of similar mechanism to that type of hypercholesteremia set in motion by the administration of these detergents. Studies concerning this point are now in progress.

Summary. Rats subjected to a procedure which substituted normal rabbit serum for their own plasma became rapidly hypercholesteremic when placed upon a high cholesterol diet.

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Complement-Fixing Murine Typhus Antibodies in Vitamin Deficiency States* IV. B_{12} Deficiency. (19887)

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The authors have presented data(1-3) concerning the effects of thiamine, pantothenic acid, pyridoxine, nicotinic acid, riboflavin and folic acid deficiencies upon complement-fixing antibody production by rats in response to a suspension of washed murine rickettsiae (*Rickettsia typhi*) as the antigenic stimulus. The present paper is concerned with the effects of vit. B_{12} deficiency upon the production of complement-fixing antibody under similar experimental conditions. The purpose of this investigation, and others in progress, is an attempt to explain the increased susceptibility and mortality that occurs in malnourished populations exposed to typhus fever(4) and other infections. Wertman and Sarandria(1) reported that pantothenic acid and thiamine deficiencies influenced the antibody response when a relatively small amount of immunizing material was injected into the rat. However, the influence was not as apparent when a relatively large amount of the same antigenic material was employed. In addition, they also reported(2) a severe impairment of antibody response in pyridoxine deficient rats when a rickettsial antigen (*Rickettsia typhi*) containing 0.0073 mg N was injected. Rats deficient in nicotinic acid did respond and produce complement-fixing antibodies when stimulated with the same amount of antigen. These same investigators also reported(3) that the riboflavin requirement for antibody production was not as critical as the folic acid requirement. The inanition control (paired-weighed) and the normal control animals (fed

* This investigation was supported by a research grant from the Upjohn Co., Kalamazoo, Mich.

ad libitum) demonstrated antibody production of comparable titers when injected with either 0.0073 mg N or 0.0365 mg N. It appeared as though the specific vitamin deficiency and not the inanition was the significant factor. Ludovici and Axelrod(5) reported no decrease in antibody production by vit. B_{12} deficient rats. A suspension of washed group O, Rh positive human erythrocytes in physiological saline was employed for both the production of circulation antibody and the hemagglutination determinations. This report was based on 7 experimental animals and only one amount of antigenic stimulation for antibody production.

Experimental. Male weanling albino rats of the Sprague-Dawley strain were housed individually in wide-meshed, screen-bottom cages and weighed daily. The room temperature was maintained at 68°F. The rats were fed a basal diet and received controlled amounts of vitamins in the form of a daily pill. The *ad libitum* and inanition controls received, in addition, 0.2 γ of vit. B_{12} (Berubigen) per day which was administered by the subcutaneous route. The basal diet was as follows: defatted, whole ground yellow corn, 45.76%; soy flour (low fat), 45.75%; Nutritional Biochemicals, salt mixture No. 2, 2%; corn oil (Mazola), 5%; l-cystine, 0.3%; choline chloride, 0.10%; i-inositol, 0.02%; p-aminobenzoic acid, 0.01%; iodinated casein (Protomine), 0.05%; 2-methyl-1,4-naphthoquinone, 0.001%; alpha tocopherol, 0.001%; and sulfasuxidine, 1%. Each of the vitamin pills supplied the following: thiamine, 80 γ; riboflavin, 120 γ; pyridoxine, 100 γ; pantothenic acid, 600 γ; nicotinic acid, 300 γ; folic acid, 10 γ; and biotin, 10 γ. Two drops of Haliver oil (3,000 USP of vit. A and 24 USP of vit. D) were administered to each rat once a week. In addition to the *ad libitum* control animals that were given the complete basal diet plus the entire vit. B complex requirement, inanition control animals were included. This was accomplished in an attempt to determine the influence of a reduced food intake upon antibody production when all the necessary B vitamins were present. Nineteen control rats were paired with 19 rats in the vit. B_{12} deficient group. When the deficient animals began to plateau or lose weight, which was approximately 3 weeks after being placed on the deficient diet, each rat of all groups was injected intraperitoneally with 0.5 cc (0.0073 mg N) of a washed, formalized suspension of *Rickettsia typhi* (murine typhus).† One cc inoculations were repeated at 7-day intervals until each rat had received a total of 2.5 cc (0.0365 mg N). The animals were bled 7 days after the first inoculation and 7 days after receiving 2.5 cc of murine typhus antigen to determine the effect of different amounts of antigenic stimulus. Rats were bled prior to starting the experiment and all sera were found to be negative when tested with murine typhus complement-fixing antigen. The complement-fixation technic employed in this study was identical to that of Plotz(6), Plotz and Wertman(7), and Wertman(8). Fixation was allowed to take place for 18 hours at 4° to 6°C. The sensitized cells (0.25 ml sheep cells 3% and 0.25 ml amboceptor containing 3 MHD) were added to each tube and incubated for 30 minutes at 37°C in a water bath. The tests were read following secondary incubation and only 4+ and 3+ fixation were accepted as the end-points. The controls necessary to insure valid results and reproducibility were included in all titrations and serum dilution tests.

Results. The vit. B_{12} control rats at the end of the feeding period had all gained weight, appeared healthy and possessed smooth even coats. The vit. B_{12} deficient rats began to lose weight and showed symptoms of the deficiency during the third week of the experiment. The members of the inanition control group were maintained at approximately the same weights as their mates in the deficient group. The initial and final average weight in grams of each group are recorded in Table I.

The end-point titers obtained in the complement-fixation test, using a suspension of murine typhus rickettsiae as the complement-fixing antigen, are likewise recorded in Table I. It appeared that a B_{12} deficiency interfered with the production of circulating anti-

† Purified antigen supplied by Dr. Herald Cox, Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.

TABLE I. Complement-Fixation Titers and Body Weight Change of Rats in a Vitamin B_{12} Deficient State.

Diet and immunization	No. of rats	Complement-fixation titers 0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	Avg body wt, g Init.	Final
B_{12} deficient													
1 inj.	38	38	—	—	—	—	—	—	—	—	—	45	112
3 "	36*	—	—	—	—	8	12	15	1	—	—		
B_{12} control (inanition)													
1 inj.	19	2	8	9	—	—	—	—	—	—	—	45	115
3 "	17*	—	—	—	—	—	—	2	8	7	—		
B_{12} control *(ad lib.)*													
1 inj.	21	2	10	8	1	—	—	—	—	—	—	45	171
3 "	21	—	—	—	—	—	—	—	5	14	2		

* Two rats died.

bodies when either a relatively small or large amount of antigenic material was injected. When a relatively small amount (0.0073 mg N) of antigenic material was employed, no detectable complement-fixing antibody was present in the sera of the deficient animals. However, the sera of 90% of the inanition and *ad libitum* controls demonstrated complement-fixing antibody when the same amount of antigenic material was employed. When a relatively large amount (0.0365 mg N) of antigen was introduced, the sera of the deficient animals did not demonstrate antibody titers as high as either the inanition or *ad libitum* control animals.

Summary. This study indicates that a vit. B_{12} deficiency impaired the production of circulating complement-fixing antibody. The sera of the inanition control and the *ad libitum* control animals possessed antibody concentrations of approximately the same titers. This was evident when either 0.0073 mg N or 0.0365 mg N was employed as the antigen. Therefore, the specific vitamin deficiency and not inanition appears to be the significant factor affecting the production of circulating complement-fixing antibody under these experimental conditions.

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Protective Effect of Adrenal Steroid Administration on Irradiated Mice. (19888)

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Altering the resistance or radiosensitivity of animals to ionizing radiation and ascertaining the physiological processes behind such resistance or radiosensitivity have been the subjects of many investigations(1-4). The idea that the condition of stress set up in animals by radiation can be altered by the administration of certain hormones has also been stud-

TABLE I. Effects of Administration of Adrenal Steroids on Mortality Rate of DBA Mice Following Radiation with 500 r Dose to the Head.

Group	Compound	Steroid dose, mg/day	Days of administration	No. of animals ♂	No. of animals ♀	Mortality at 45 days ♂	Mortality at 45 days ♀
I*	C†	1	3 prerad.	12	12	0	0
II*	C	1	3 postrad.‡	8	8	4	0
III (control)	—		Irrad. only	12	12	12	12§
IV "	C	1	3 (no irrad.)	11	11	0	0
I*	DCA	.5	3 prerad.	12	12	0	0
II*	DCA	.5	3 postrad.‡	6	6	0	0
III (control)	—		Irrad. only	13	13	13	13
IV "	DCA	.5	3 (no irrad.)	11	11	0	0

* Although 45 days is indicated, these treated animals were still surviving and apparently well after 4 months.

† C = cortisone.

‡ Mice received cortisone or DCA immediately after radiation.

§ Two lived 52 days rather than 45 days after radiation.

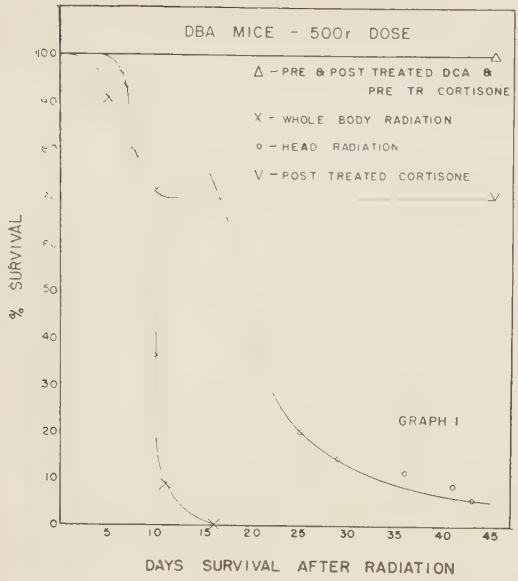
ied(5-7). Moreover, a direct relationship between the adrenal cortex and resistance to whole-body radiation has been postulated, but this postulate has been neither proved nor rejected. Therefore, it is the purpose of this study to endeavor to investigate the role of the pituitary-adrenal axis in radiosensitivity or resistance in mice or, more specifically, to determine the effect of cortisone and DCA as agents in minimizing the radiosensitivity in mice as a result of ionizing radiation.

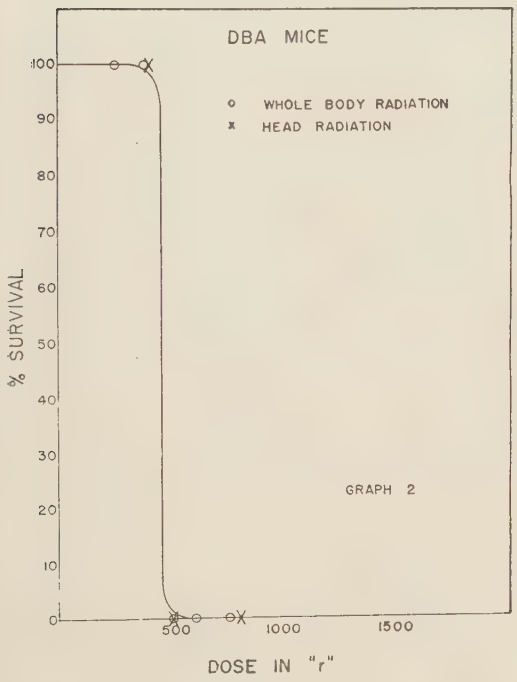
Materials. a. *Mice.* The mice used in these experiments were all from our own inbred strain of DBA mice. Both males and females, having an average age of 6 weeks and an average weight of 20 g, were used. Purina fox chow and water were available at all times. b. *Hormones.* The hormones used in these experiments were 11-dehydro-17 hydroxycorticosterone-21-acetate (Cortisone Acetate—Merck) and desoxycorticosterone acetate (Doca Acetate—Organon). c. *Radiation.* The x-rays used had a H.V.L. of 0.9 mm copper with a dose rate of 150 r/min. (air) at a distance of 30 cm. *Procedure.* The lethal dose curves for DBA mice with head and whole-body radiation were first established (Graph 2), and from these curves a dose of 500 r (air) was selected for radiation of the head. This dose produces death in 100% of the unprimed animals. Animals were primed to observe whether or not the aforementioned hormones would afford protection when a 500 r dose was administered to the head. In a subsequent paper lethal dosage curves for head radiation, whole-body radiation, and whole-body-except-head radiation for this (DBA) strain and other strains of mice will be presented. Preliminary data obtained to date show that there is a marked strain difference in the response to ionizing radiation. As shown in Table I, the mice were divided into 4 groups. The first group received injections of hormones prior to radiation, the second group was treated subsequent to the radiation, the third (control) group received radiation only, and the fourth (control) group received hormones only.

Results. It is evident from Table I that, whereas a dose of 500 r produced death in all of the control animals (group 3), those animals which had been fortified with hormones prior to irradiation were apparently unaffected, as judged by the mortality rate. The second group of mice, namely those which received the hormones subsequent to irradiation, also showed evidence of a protective effect of the hormones, but to a lesser degree than the first group which received the hormones prior to irradiation.

In order to further analyze the data with respect to time of survival, the results are plotted in Graph 1. The data for whole-body radiation with a dose of 500 r are included.

When a 500 r dose is delivered either to the whole body or to the head, a 100% mortality rate is obtained (Graph 2); however, mice receiving whole-body radiation succumb sooner than those receiving head radiation only. Over 200 mice were utilized to establish the





fact that a 500 r dose to the whole body is fatal.

Discussion. The foregoing data clearly indicate that administration of the gluco-corticoid, 11-dehydro-17-hydroxycorticosterone 21-acetate, or the minero-corticoid, desoxycorticosterone acetate, enhances the survival rate of DBA mice when radiated with a 500 r dose to the head. This investigation can be compared to investigations made by others despite the fact that other workers used whole-body radiation and different dose levels, hormones, strains of mice, and even different animals. Therefore, looking at the results of other investigators only on the basis of whether or not the hormones used afford protection against ionizing radiation, and yet not minimizing the importance of differences in strains, hormones, radiation dosages, etc., one can probably say that our findings agree with those of Ellinger (7), who has reported that 0.5 mg of desoxycorticosterone given after radiation reduces the mortality rate. However, these findings are in contradistinction of those of Straube *et al.*(8), and Graham *et al.*(9), who were unable to demonstrate a significant effect from DCA. Smith *et al.*(10) report the failure of cortisone or ACTH to reduce the mortality in irradiated mice. In our work with DBA irradiated mice, cortisone was shown to be effective. A strain difference in the metabolic handling of these compounds or variations in the effective dosage levels given may be significant factors in accounting for the apparent inconsistencies reported in the literature.

In this study a correlation is seen between whole-body and head radiation (Graph 2), for similar mortality levels are obtained in both cases with a 500 r dose. However, the mice receiving whole-body radiation succumb within 16 days, whereas the irradiated mice receiving 500 r to the head succumb around the 45th day (Graph 1). The more rapid death rate for whole-body irradiated mice may be due to a more rapid failure of functional reconstitution of one or more of the tissues or organs in the body, *e.g.*, the hematopoietic system and the endocrine system. From this difference in mortality time in our data, one can suppose that radiating just the head region involves a less direct impairment of radio-sensitive organs, *e.g.*, spleen, bone marrow(11), and adrenal glands(12). However, since the pituitary-adrenal axis is known to play an important role in metabolic activities, the irradiated mice receiving head radiation succumb eventually, possibly as a result of injury to pituitary cells which disturbs the elaboration of metabolic hormones required to

support normal metabolic activity. In other words, the pituitary gland, particularly the anterior lobe, is profoundly influenced by ionizing rays. As a result of this radiosensitivity along with other disfunctions, a state of adrenal insufficiency is established. From this one can assume that the extent of pituitary damage determines the degree of adrenal insufficiency. The fact that both cortisone and DCA are able to increase the survival time in DBA mice suggests that in this strain a state of adrenal insufficiency is avoided either by rendering the pituitary less sensitive to ionizing radiation or by preventing an early decrease in adrenal-cortical activity after irradiation, which, if prolonged, may possibly result terminally in a relative adrenal insufficiency and finally death.

Summary. 1. Young male and female DBA mice were exposed to 500 r head radiation or whole-body radiation. A correlation is observed between head and whole-body radiation, as judged by the mortality rate. In both instances a 500 r dose gives a 100% mortality rate, but mice receiving whole-body radiation succumb sooner. 2. Mice receiving 500 r head radiation and primed before or after radiation with cortisone or desoxycorticosterone showed evidence that both of these compounds afford protection from the lethal effect of ionizing radiation. 3. Interpretations of the role of the pituitary-adrenal axis under the conditions of these experiments are discussed.

We wish to thank Dr. Kenneth Wade Thompson, Director of Medical Research, Organon Inc., for the generous donation of desoxycorticosterone acetate (Doca).

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Stability of Mumps Antibody and Antigen at Various Temperatures.* (19889)

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During the course of an investigation on the antibody response of man to vaccination with the mumps antigen it became necessary to obtain information as to the stability of the mumps antibody and antigen. The stability of the complement-fixing (CF) antibody of immune sera was tested in relation to temperature variations during inactivation prior to the CF test, and following storage. The stability of the CF antigen and the CF antibody-producing antigen of a commercially prepared vaccine were also tested.

Materials and methods. The complement-fixation test. The test employed in these studies was developed by Habel(1). Commercial hemolysin was used in the amount of 2 units in 0.2 ml. Lyophilized guinea pig comple-

* This study was supported in part by a grant from Eli Lilly and Co., Indianapolis, Ind.

ment† was diluted to contain 1⅓ units in 0.2 ml. Defibrinated sheep red blood cells (SRBC) were used as a 2% suspension in 0.85% NaCl solution; the salt solution also served as the diluent in the test. Depending upon which was being tested, serial 2-fold dilutions of the antigen or antibody were made, the lowest dilution being 1:2 in 0.2 ml unless otherwise mentioned. Four CF units of antigen or antiserum (depending on which was being titered) and 1⅓ units of complement, each in 0.2 ml, were then added to all tubes except the controls. The tubes were incubated for one hour in a waterbath at 37°C. After adding 0.2 ml of a 2% suspension of SRBCs plus 2 units of hemolysin in 0.2 ml, the tubes were again incubated at 37°C for one hour and placed in a refrigerator at 5°C; the results were read after 18 hours. The endpoint of the reaction was taken as the greatest initial dilution of test material causing 4+ fixation. All tests were run in duplicate. *Storage of stock antiserum.* The pooled mumps convalescent serum, which was obtained from the Michael Reese Serum Center, Chicago, was transferred aseptically to chemically clean glass ampules in approximately one ml amounts. The ampules were flame-sealed and stored at —15°C until tested. *Determination of the antigenicity of the vaccine.* In testing the antigenicity of the vaccine, 450 g male guinea pigs were employed. An adjuvant was used after the procedure of Freund (2). The proportions were: 2 parts of mumps vaccine, 1 part Falba, and 2 parts paraffin oil containing heat-killed *M. tuberculosis* mixed well immediately before injection by forcibly filling and emptying a syringe with the material. *Preparation of mumps vaccine.* The vaccine‡ was a suspension of an alcohol concentrated, ultra-violet light inactivated mumps virus (Enders and Habel strains) which had been grown in embryonated eggs. The antigen was suspended in 0.85% NaCl solution with "Merthiolate" (Thimerosal, Lilly) 1:10,000 and formalin 1:1,000 added as preservatives. Sterility and antigenicity tests were made according to specifications of the National Institutes of Health of the United States Public Health Service.

Results. Heat-stability of the mumps CF antibody. In performing the CF test the practice has been to inactivate the serum at 56°C to destroy the complement; however, it is now recognized that cross reactions with other antigens occur after inactivation at 56°C and that these can be eliminated by inactivation at 60°C. In an effort to determine the stability of mumps antisera at higher temperatures, samples were heated at different temperatures for varying periods of time. Five one ml samples of undiluted antisera were placed in waterbaths ranging from 48 ± 0.5°C to 64 ± 0.5°C in increments of 4°C for 30 minutes; one other sample remained at room temperature. In 4 experiments no decrease in CF titer was noted between 22° and 60°C. Heating the antisera for 30 minutes at 64°C coagulated them and rendered titration impossible.

The stability of the antibody on storage at —15°C. During the course of 2 months the sera were stored at —15°C in sealed ampules and the titer remained 1:80.

The effect of simulated shipping conditions on the CF titer of antisera. An effort was made to determine the effect of sending antisera from the point of collection to the laboratory upon the determination of antibody levels. One series of samples of antisera was refrigerated for 7 days in an insulated box containing dry ice, the temperature gradually rising from —35° to —27°C as the CO_2 sublimated. Three samples were stored unfrozen: one at 5°C to approximate cold weather conditions, one at room temperature (22° to 30°C) for moderate weather, and one series at 37°C to simulate warmer weather. All samples were titered each day during a period of 7 days. There was no loss in CF titer under these conditions. Those samples stored at room temperature and at 37°C became anticomplementary within one week, but this condition was corrected by inactivating the serum at 60°C for 25 minutes.

Having determined the stability of the antibody under the conditions tested, it seemed advisable to determine the effects of various

† Processed by Carworth Farms, New City, N. Y.

‡ Kindly supplied by Eli Lilly and Co., Indianapolis, Ind.

TABLE I.
Effect of Storage at Various Temperatures on Complement Fixation Titer of Mumps Vaccine.

	Reciprocal of mean titer				
	Temp., °C				
Time, wk	−70	−15	5	22 to 30	37
0	32	32	32	32	32
1	16	8	16	32	32
2	8	4	32	32	32
4	4	<2	32	16	32
5	2	2	32	16	16
6	2	<2	32	16	16
7	2	<2	32	16	16

periods of exposure at different temperatures on the antigenicity of the vaccine.

Stability of the mumps CF antigen under conditions of prolonged storage at 5°C. Three samples which had been stored in an electrical refrigerator at approximately 5°C for 3 years were titered. The CF titer of the preparations had been 1:16 when first titered by the same procedure in another laboratory 3 years previously, and the titer was maintained over the 3-year period. It is possible that a decrease in titer might have been masked by differences in technic between the individuals who made the initial and subsequent titrations. The stored vaccine was slightly anticomplementary, but this was evident only in the lowest dilution (1:2) of the antigen.

Stability of the mumps CF antigen at various temperatures. Since vaccines may be subject to various temperatures for varying periods of time before use, an effort was made to determine the effect of different temperatures on the CF titer of the mumps vaccine. Fifty ml of vaccine were pooled and distributed in approximately 10 ml amounts in corked and paraffin-sealed tubes and stored at —70°, —15°, 5°, 22-30° (room temperature) and 37°C for a 7-week period. Antigens were titered in duplicate by the CF test at weekly intervals with the exception of the third week. All samples stood at room temperature for one hour before each titration in order to equalize their temperatures. It was found that the vaccine was quite stable except at —70° and —15°C. The vaccines stored at —15° and at —70°C rapidly lost titer (Table I).

In order to determine more accurately the temperature at which alteration of the CF antigen occurred, a different range of temperatures was employed. Six 10 ml samples were stored at —70°, —23°, —15°, —10°, —5°, and 5°C. Each sample was then titered in duplicate at weekly intervals. Storage at —15°C or lower caused a loss of CF titer to less than 1:2 within one week, while storage at —10°C and —5°C effected a loss to less than 1:2 by the end of the third week. Storage at 5°C had no effect on the titer of the vaccine.

In an effort to discover if the loss of titer in the vaccine which had been stored at subfreezing temperatures was due only to the effects of repeated freezing and thawing, which was necessary in the course of the weekly titrations, 3 ml samples were subjected to the effects of repeated temperature changes which ranged from (a) —15°C to thawing at 5°C, (b) —15°C to thawing at room temperature, and (c) —15°C to thawing at 37°C. The samples were titered in duplicate after having been frozen and thawed 5 times and after repeating the procedure 10 times. None of the various freezing and thawing procedures had any effect upon the titer of the vaccine; it remained 1:32 throughout the experiment.

The loss of ability of the stored vaccine to stimulate the production of CF antibody. It was decided to test the ability of vaccine which had lost its CF titer upon storage at subfreezing temperatures to stimulate the production of CF antibodies in guinea pigs. The use of this vaccine alone failed to elicit any antibody response, so an adjuvant was employed to enhance its antigenicity. Accordingly, 0.4 ml of vaccine the titer of which had dropped from 1:32 to less than 1:2 was mixed with 0.6 ml of Freund's adjuvant(2) and injected subcutaneously into 5 male guinea pigs. Five animals were also injected with 0.4 ml of a similar preparation having a CF titer of 1:32. All animals were bled from the heart prior to injection, the serum collected and stored at —15°C until all samples could be titered on the same day. Blood was again removed from all animals 17 days after injection and all of the sera were titered for the presence of CF antibodies.

From Table II it can be seen that the vac-

TABLE II. Production of Complement Fixing Antibodies in Guinea Pigs upon Injection of Vaccine Which Had Lost Its Complement Fixation Titer upon Storage at −15°C.

Guinea pig	Substance inj.*	Reciprocal of titer at 16 days
1	Adjuvant + vaccine with	256
2	1:32 CF titer	512
3		512
4		512
5		256
	Mean	410
6	Adjuvant + vaccine with	64
7	<1:2 CF titer	64
8		64
9		64
10		128
	Mean	77

* .6 ml adjuvant + .4 ml vaccine.

cine which had lost its CF titer upon storage at —15°C was capable of stimulating the production of CF antibodies in guinea pigs, but such antibody levels were only about 1/5 of those produced by vaccine with a CF titer of 1:32. Essentially the same results were obtained when a vaccine with a titer of 1:8 was used.

Discussion. Our results indicated that the CF antibodies in mumps immune sera were not affected by heating at 60°C for 30 minutes. Thus it appears that the mumps antibody is as stable as the antibody against a number of other viruses. The stability of CF antibody for as long as a week at temperatures of —35°C to +37°C indicated that titers obtained on sterile sera which have been shipped and stored under these conditions would not have changed appreciably since the time the samples were taken.

When the mumps vaccine was stored at 5°C for 3 years it apparently lost no CF titer. It would therefore seem that storage at refrigerator temperature for one year would allow a wide margin of safety.

On storage at freezing temperatures from —5° to —10°C the CF titer and the CF stimulating capacity of the vaccine disappeared or greatly diminished. Thus it seems that storage at freezing temperatures would render a vaccine useless in one week. These results were similar to those of Penttinen(3), Stanley (4), and Salk(5).

The use of an adjuvant with the vaccine in order to obtain detectable or higher levels of CF antibodies is in agreement with the findings of Enders and coworkers(6), Salk(7), and Habel(8).

Summary. 1. The CF antibodies of pooled mumps antisera were stable for 2 months at —15°C, and for 30 minutes at 60°C. 2. Subfreezing temperatures had a markedly deleterious effect on the CF titer of mumps vaccine; temperatures above freezing had little effect; samples stored at 5°C for 3 years showed no drop in titer. After as many as 10 cycles of freezing and thawing, the CF titer of the vaccine remained unchanged. 3. Vaccine which had lost its CF antigen was able to stimulate the production of CF antibodies in guinea pigs provided Freund's adjuvant was used; however, the order of response was approximately 1/5 that produced by the same vaccine with the CF antigens intact. 4. The significance of these observations is discussed in relation to mumps vaccination studies.

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Rate of Absorption and Formation of Liver Glycogen by Glycine.* (19890)

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The rate of absorption of glycine from the gastrointestinal tract of the white rat has been studied by Wilson and Lewis(1), Wilson (2,3), and Butts, Dunn, and Hollman(4). Application of the Van Slyke amino nitrogen method gave rates varying from 50 to 75 mg per 100 g per hour for free glycine and from 30 to 90 mg for the half sodium salt.

Wilson and Lewis(1) were unable to find any increase in liver glycogen after feeding glycine and Wilson(2) found a slight increase in 3 hours and substantially the same value in 16 hours. Butts *et al.*(4) reported a definite increase after 8 hours while Stöhr(5) found variable results. Mackay, Wick, and Carne (6) found that glycine formed glycogen rather slowly, the maximum value was 2.36% after 14 hours and a smaller peak occurred at 4 hours. Olsen, Hemingway, and Nier(7) fed glycine with the carboxyl carbon labeled with the stable isotope, to mice and found the peak of glycogen formation to be at 14 hours. Preliminary to a study of the effect of cortisone on liver glycogen formation following the feeding of glycine we have investigated its rate of absorption and capacity to form liver glycogen.

Experimental. White rats, weighing 100 to 150 g, fasted for 48 hours, were fed the amino acid by stomach tube. Glycine, dissolved in water, was administered at levels of 100, 150, 300 and 450 mg per 100 g of body weight for 0.5, 1, 2, and 3 hour absorption periods, respectively. For the glycogen studies for periods longer than 3 hours 450 mg per 100 g was used.

At the end of the absorption period the animals were sacrificed and the entire gastrointestinal tract and the liver removed. The gastrointestinal tract was ground in a Waring blendor with 50 ml of a 10% aqueous solution of trichloracetic acid, the extract filtered with suction through a Celite pad and aliquots of the filtrate taken for analysis. The glycine was determined by the specific method of Alexander, Landwehr, and Seligman(8), all the absorption results are corrected for the loss of 3.6% that occurred in the procedure. Confirmatory values in excellent agreement with the colorimetric values were obtained by the amino nitrogen method of Pope and Stevens (9) which we have used previously in similar studies(10). The formation of liver glycogen was followed using the method of Good, Kramer, and Somogyi(11). The glycogen values for periods of time longer than 3 hours are averages of determinations on from 4 to 6 animals.

TABLE I. Rate of Absorption of and Glycogen Formation by Glycine.

No. of animals	Time, hr	Rate, mg/100 g/hr	Glycogen, %
6	.5	96.9 ± 6.6*	.34†
8	1	111 ± 8.8	.53
6	2	102.5 ± 7.1	.29
6	3	94 ± 4.9	.46
Avg 26		102.1 ± 6.8	

* Stand. dev.

† The glycogen content of livers of 12 control rats averaged .04%.

Glycine was absorbed (Table I) at a faster rate than reported by Wilson and Lewis(1) and somewhat faster than found by Wilson (3) after a 2-hour absorption period. The rate is approximately the same as reported by Wilson(3) for the half sodium salt, we conducted several experiments with the half sodium salt and found its rate of absorption to be somewhat less than that of the amino acid itself. Glycine is definitely glyconeogenic giving a peak value for liver glycogen at 14 hours (Fig. 1). A peak at one hour was noted, Mackay *et al.*(6) had found a peak at 4 hours, these early peaks may represent cortical stimulation.

Summary. The rate of absorption of gly-

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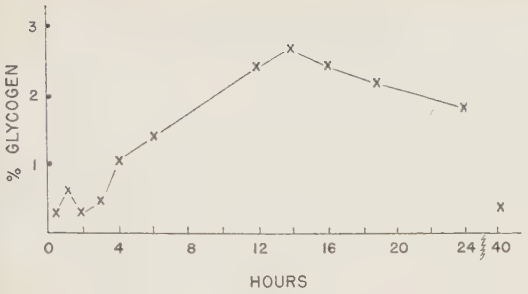

FIG. 1.

cine from the gastrointestinal tract of the white rat was found to be 102 g per 100 g of body weight per hour. The rate did not vary significantly during absorption periods of from 0.5 to 3 hours. Glycine is definitely glyconeogenic reaching a peak of glycogen production 14 hours after its administration. A smaller but definite peak was observed at one hour.

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Effect of High Fat Diet on the Potassium Deficiency Syndrome in the Rat.* (19891)

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Potassium deficiency has been studied by a number of workers and physiological and pathological effects of the deficiency have been described in rats, mice, dogs, and calves(1). In a previous experiment it was observed(2) that when rats were fed potassium deficient diets containing 5% and 20% corn oil, the resulting deficiencies were more severe when the diet contained 20% corn oil. Since these diets were fed *ad libitum*, the observed results could have been due to decreased feed and potassium consumption by the rats receiving the 20% corn oil diet. The experiment herein described was designed to determine if this were the case, or if the higher level of fat in the diet increased the rat's need for potassium.

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TABLE I. Percentage Composition of Diets.

	Diet 1	Diet 2	Diet 3	Diet 4
Sucrose	39.2	73.2	39.2	73.2
Casein*	19.3	19.3	19.3	19.3
Corn oil	20	5	20	5
Na and K free salts	2.5	2.5	2.5	2.5
$NaHCO_3$	.91	.91	.91	.91
$KHCO_3$	.08	.08	.08	1.28
Vitamins†	.053	.053	.053	.053
Choline	.1	.1	.1	.1
Cellu-flour‡	19	—	19	—

* GBI-vit. free.

† Vitamins—mg/kg of diet: thiamine, 3; riboflavin, 3; pyridoxine, 2; Ca-pantothenate, 20; inositol, 100; niacin, 20; para-aminobenzoic acid, 250; biotin, .1; folic acid, .25.

‡ Chicago Dietetic Supply House, Chicago, Ill.

Experimental procedure. Six 40-50 g weanling male albino rats of the Sprague-Dawley strain were placed on each of the 4 diets given in Table I. The salt mixture employed was that used by Grunert and Phillips(3) with sodium and potassium added as the bicar-

TABLE II. Summary of Results.*

	Lot 1 (.03% K, 20% corn oil)	Lot 2 (.03% K, 5% corn oil)	Lot 3 (.03% K, 20% corn oil)	Lot 4 (.5% K, 5% corn oil)
Skeletal muscle				
meq. % Na	6.55 ± 1.3	5.22 ± 1.1	5.30 ± 2	1.94 ± .42
meq. % K	5.48 ± 1.7	6.40 ± .2	5.56 ± .8	9.35 ± .68
Kidney				
mg fresh wt/100 g body wt	1282 ±160	1274 ±67	1433 ±268	567 ±21
mg dry wt/100 g body wt	224 ± 25.6	242 ±24	239 ± 11	135 ±10
% water	82.5 ± .4	80.8 ± 1.7	83 ± 2.1	75.9 ± .6
Myocardial necrosis and fibrosis†	+ + +	— + —	+ + +	— — —
	+ +	— — —		— — —
Dilatation of renal tubules†	+ + +	— + —	+ + +	— — —
	+ +	— — —		— — —

* Stand. dev. are given.
† Each + indicates an animal in which the specified lesion was moderate or extensive.
Each — " " " " " " " " " slight or absent.

bonate when needed. Diets 1, 2, and 3 contained 0.25% sodium and 0.03% potassium and diet 4 contained 0.25% sodium and 0.5% potassium. Diets 1 and 3 were made isocaloric with diets 2 and 4 by the addition of cellu-flour as indicated. The rats which received diets 1 and 2 were pair-fed throughout the experimental period and diets 3 and 4 were fed *ad libitum*. Thus, the dietary intake of calories and of potassium for each of the rats receiving diet 2 was the same as that of its diet 1 pair mate. The original plan was to maintain the rats on the experimental diets for six weeks. Death of one diet 1 and three diet 3 rats on the twenty-fourth day of the experiment, however, prompted the termination of the experiment at that time. The deaths of these animals appeared to be due to obstruction of the large intestine by impaction of large masses of cellulose. The surviving animals were killed with natural gas, autopsied, and specimens were removed for sectioning and sodium, potassium, or water analyses. Heart and kidney sections were fixed in Bouin's fluid, embedded in paraffin, and stained routinely with hematoxylin and eosin for histological study. The left kidney of each rat was weighed to the nearest 0.2 mg on a Roller-Smith torsion balance and analyzed for moisture by drying to constant weight. Specimens of skeletal muscle were analyzed for sodium and potassium with a Perkin-Elmer Model 52A flame photometer after wet ashing.

Results and discussion. There were no statistically significant differences in the sodium or potassium concentrations in the striated muscle or in the per cent water in the kidneys of the rats on the 3 potassium deficient diets (Table II). There was a trend, however, toward more sodium and less potassium in the muscle sample, and increased water in the kidneys of the rats receiving diet 1 (.03% K, 20% corn oil) as compared with the values for the diet 2 (.03% K, 5% corn oil) animals. The animals on all 3 of the potassium deficient diets had greatly reduced potassium and increased sodium in their skeletal musculature and greater fresh and dry kidney weights per 100 g of body weight when compared with their respective controls. All of these effects of potassium deficiency except the increase in dry weight of kidney per unit of body weight are well known.

Examination of the heart and kidney sections revealed that the rats which had received diet 1 (.03% K, 20% corn oil) had much more extensive myocardial fibrosis and renal tubule dilatation than the rats which received diet 2 (.03% K, 5% corn oil). All of the surviving diet 1 rats had moderate to extensive myocardial fibrosis and kidney tubule dilatation, while only one of the diet 2

animals had myocardial or renal pathology that could be considered to be more than slight.

The reason for these results is not known. Rix and Ebrhardt(4) observed fluctuations in the potassium and calcium concentrations in the blood sera of rabbits when linseed oil was added to the rabbits' hay and turnip diet as daily 5 cc doses. They believed that the increase in serum potassium which they observed during the first 6 weeks of linseed oil administration was due to increased absorption of potassium from the intestine. They, however, were unable to explain the rapid fall in serum potassium concentration which occurred in their rabbits after the first 6 weeks on this regimen. The potassium content of the diet which they employed was not stated.

Summary. A diet containing 0.03% potassium and 20% corn oil produced a greater incidence and extent of the myocardial necrosis and fibrosis and dilatation of renal tubules typical of potassium deficiency than did an isocaloric, 5% corn oil diet containing the same amount of potassium when the 2 diets were pair fed. No statistically significant differences were observed in other criteria of potassium deficiency.

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Evidence for an Unidentified Growth Factor Required by *Microbacterium flavum.** (19892)

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(Introduced by George M. Briggs.)

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Doetsch and Pelczar(1) reported that several strains of *Microbacterium lacticum* would grow in a medium consisting of vitamin-free acid-hydrolyzed casein, inorganic phosphates, calcium D-pantothenate and thiamin hydrochloride. Calcium D-pantothenate was shown to be essential for growth and thiamin to be stimulatory. The same workers(2) reported similar results when 18 amino acids were substituted for the casein hydrolysate. Supplementation of this latter medium with riboflavin, niacin, pyridoxine, biotin, folic acid, and glutamine produced growth comparable to that obtained in trypticase soy broth. *Microbacterium flavum,* however, was unable to grow in any of the synthetic media prepared. Bishop *et al.*(3) substantiated these results with *M. flavum* and found that addition of crude materials such as phytone, yeast extract, proteose-peptone No. 3, Wilson's Liver L, and asparagus juice resulted in media which supported good growth of *M. flavum.* The data indicated that a combination of substances was active in stimulating optimum growth. A further study of the nutritional requirements of this organism is the subject of this report.

Methods. Microbacterium flavum, OJ8, (ATCC 10340), was maintained on slants of Difco Micro Culture Assay Agar. Subcultures were made weekly, incubated for 24 hours at 30°C and then stored in the refrigerator. Inocula were prepared by transferring the stock cultures to 10 ml of Difco Micro Inoculum Broth and incubating at 30°C for 24 hours. The culture was centrifuged, washed

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† Merck Research Fellow.

TABLE I. Effect of EDTA and $MgSO_4$ on Growth of *M. flavum*.

$MgSO_4$, mg/tube	Galvanometer readings	
	No EDTA	300 μg EDTA/tube
0	94	100
1	81	100
5	67	85
10	62	76.5
50	56.5	68
100	56	63.5
200	56	
500	80	100

twice with physiological saline, resuspended in 10 ml of saline and adjusted by dilution to a reading of 65 on the Evelyn Photoelectric Colorimeter, using the 515 mμ filter. One drop of a 1:100 dilution of this suspension was used to inoculate each tube of experimental media. The basal medium employed was that of Doetsch and Pelczar modified by omission of glutamine, hydroxy-L-proline and DL-threonine and addition of aspartic acid (0.05%) (3), and $MgSO_4 \cdot 7\ H_2O$ (0.0033%). Samples of materials tested for growth response were added to 20 × 150 mm tubes in the desired amounts in duplicate, the volume made to 3 ml with distilled water and 3 ml of the medium (double strength) added, giving a total volume of 6 ml per tube. These tubes were then capped with glass hats, autoclaved for 12 minutes at 120°C, cooled and inoculated. They were incubated at 30°C for 36-38 hours and were agitated daily to facilitate reading by breaking up the clumps of bacteria. The amount of growth was determined by means of the Evelyn Photoelectric Colorimeter using the 620 mμ filter. Final pH and acid titration of individual tubes did not follow dosage response patterns. A standard reference material was prepared by hydrolyzing soy bean oil meal (Archer-Daniels-Midland Co.) with 12 N HCl, adsorbing with Norit at pH 3.5 for 6 hours (on the basis of 1 g of Norit to 1 g of dry hydrolysate), and neutralizing. The filtrate standard was designated S3, and 1 mg was arbitrarily assigned one unit. A dosage response curve was obtained with 2 to 100 mg S3 per 6 ml of medium. A similar fraction, P3, was prepared from Alpha protein (The Glidden Co.), a com-

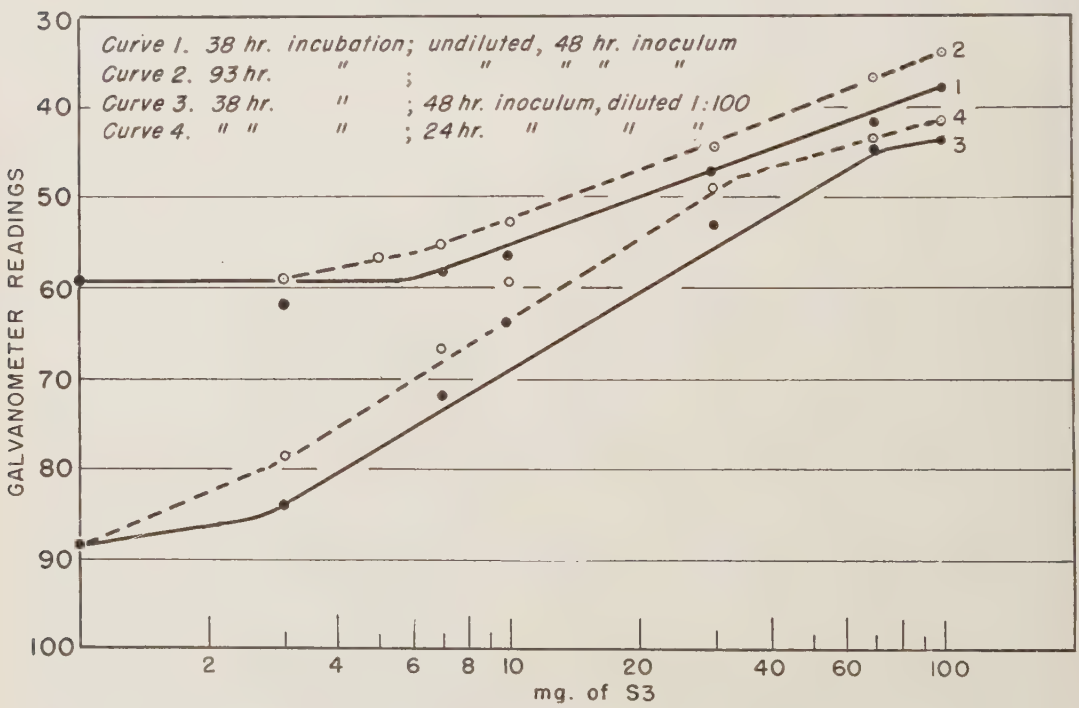

FIG. 1. Effect of varying inoculum and incubation time on growth response curves for S3 in the presence of $MgSO_4 \cdot 7H_2O$.

mercial preparation of the protein portion of soy bean meal.

Results and discussion. In preliminary work the testing of a variety of compounds indicated a requirement for minerals. Consequently $NaSiO_3$, $CaCl_2$, Na_2SO_4, NaCl, $MgSO_4$, $FeSO_4$, $SrCl_2$, $MnSO_4$, and Na_2SO_3 were tested for activity in the medium lacking both $MgSO_4$ and a source of S3 and with a 48-hour inoculum merely adjusted to 65 on the colorimeter without the 1:100 dilution. Only $MgSO_4$ stimulated growth and that only to the half-maximum point. Addition of EDTA (ethylene diamine tetraacetic acid, a solubilizing chelating agent) caused complete inhibition which was overcome by addition of $MgSO_4 \cdot 7\ H_2O$ (Table I). Thus $MgSO_4$ was included in the medium in the concentration indicated above. The assay procedure was further improved by reducing the inoculum growth period from 48 to 24 hours, reducing the assay incubation time from 96 to 48 hours and by diluting the inoculum 1:100. The effects of these are shown in Fig. 1, using S3 as the sample. It will be noted that little growth was obtained when no sample was added, steeper curves resulted on addition of S3 and straight line responses were easily obtained on semi-logarithmic graphs when the inoculum was diluted 1:100. The growth response obtained with the inclusion of optimal amounts of S3 in the medium was comparable to that obtained with trypticase soy broth or Difco Micro Inoculum Broth.

Using the improved medium and inoculum, a recheck of the source materials previously tested (*v. supra*) showed that Wilson's liver L, asparagus juice, P3 and yeast extract were still active while phytone, though stimulatory, was less active than before (Table II). This indicated that part of the original activity of phytone was probably due to the presence of magnesium. The lack of complete growth at high levels of phytone may be due to a high magnesium content and resultant toxicity (Table I), or the higher activity of the other materials may be due to a larger concentration of a secondary growth-stimulating factor.

Since good responses were obtained with the hydrolyzed protein, S3, and since S3 was Biuret negative, 20 amino acids individually and in several combinations and vitamin-free casein hydrolysate were tested for effect. None of these stimulated growth in the presence or absence of S3. This is not surprising since P3, the preparation from Alpha protein, had only 40% the activity of S3.

TABLE II. Potencies* of Source Materials for *M. flavum*.

Sample	Units/mg
Phytone	.3
P3†	.4
S3†	1
Yeast extr.	1
Liver L	2.8
Asparagus juice	3.8

* 1 unit = 1 mg of the Norit filtrate of the HCl hydrolysate of soy bean meal.
† See text for description.

The active factor(s) could not be replaced by the *Leuconostoc citrovorum* factor, a *Lactobacillus bulgaricus* factor concentrate, vit. B_T concentrate, fraction A (a peptide fraction from hemoglobin isolated by Kao, unpublished), orotic acid, vit. B_{12}, biocytin, cytidylic acid, xanthine, adenine, guanine, uracil or a combination of the last three. The factor(s) is not strepogenin since it is not a peptide, it withstands HCl hydrolysis(4) and activity remains even after 4 Norit adsorptions, which procedure removes strepogenin(5).

Other characteristics of the factor(s) found in S3 are stability to treatment with HNO_2 and H_2O_2, appearance on both sides of a dialyzing membrane with slightly more activity residing in the residue and separation from aqueous solution by acetone. After addition of only a small amount of acetone an immiscible brown liquid which contained most of the activity appeared at the bottom of the container. Even though there was a decrease in total weight there was not a proportionate increase in activity, possibly indicating the presence of an inhibitor.

Studies with paper partition chromatography indicated that further purification and concentration were necessary since the large sample required to obtain a growth response caused streaking of the chromatograms.

Summary. *Microbacterium flavum* has been shown to require an unidentified factor(s) and

magnesium for optimal growth in a synthetic medium. The addition of this substance to an otherwise synthetic medium supports growth approximately equivalent to that obtained in Difco Micro Inoculum Broth or trypticase soy broth. The unidentified factor(s) can be found in Wilson's liver L, asparagus juice, yeast extract, soybean meal, and Alpha protein. It is not replaceable by any known growth factors tested. It is stable to HCl hydrolysis, treatment with HNO_2 and H_2O_2 and is separated from aqueous solution with acetone.

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Intestinal Absorption of Glucose in Hypothalamic Obesity. (19893)

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The demonstration that the obesity which often appears in rats as a result of damage to the hypothalamus is primarily due to hyperphagia(1) has not entirely dispelled the traditional concept that this disorder may be a manifestation of some disturbance of metabolism. Among the more commonly indicted metabolic processes are those involved in intestinal absorption. Soulairac(2) has reported that hypothalamic lesions in rats may elevate the rate of glucose absorption from the intestine. Unfortunately, his data are too few for statistical analysis, and although he claimed that changes in the rate of glucose absorption run parallel with changes in the appetite for food (and specifically for glucose) he failed to show that the animals in which he found an increased rate of glucose absorption (10 days after placing the lesions) were either hyperphagic or obese. The relationship of Soulairac's finding to hypothalamic obesity, therefore, appears doubtful. On the other hand, Brooks(3) concluded, on the basis of indirect evidence (food-feces ratios), that the rate of intestinal absorption of food in rats with hypothalamic obesity was not elevated above that in controls.

The present investigation was undertaken since direct observations of the rate of glucose absorption in rats with clearly established hypothalamic obesity have not heretofore been reported.

Experimental. Large bilateral electrolytic hypothalamic lesions were placed in a series of male rats (of both Sprague-Dawley and Long-Evans strains, weighing between 220 g and 300 g) using the Krieg-Johnson* stereotaxic apparatus. The rats were operated upon in several groups, a few non-operated rats from each group being reserved as controls. The animals were then kept in individual cages for periods ranging from 29 to 47 days and fed Rockland chow pellets *ad libitum*. Twenty-one rats were used for glucose absorption determinations: 10 unoperated controls, 9 with hypothalamic obesity (HO), and 2 HO thyroxinized.† In addition, 3 unlesioned rats were thyroidectomized (3-4 weeks prior to use) and five were rendered hyperthyroid† to serve as method controls. The determination of glucose absorption was carried out on anesthetized rats exactly as described by Bogdanove and Barker(4). Following a 48-hour fast the rats were given nembutal (45 mg/kg)

‡ Present address: National Cancer Institute, Bethesda 14, Md.

* Obtained from the Johnson Scientific Co., Berwyn, Ill.

† Thyroxine was injected subcutaneously in 200 μg doses thrice daily for 7 to 11 days.

TABLE I. Glucose Absorption by Control, HO, Thyroxinized HO, Thyroidectomized and Thyroxinized Unlesioned Rats.

	No. of rats	GCA*	Range	S.E.
Controls	10	114.0	74-162	±9.60
HO	9	107.4	93-132	±3.82
HO thyroxinized	2	173.5	163-184	—†
Thyroxinized	5	191.6	170-204	—†
Thyroidectomized	3	75.2	63-86	—†

* Glucose coefficient of absorption = glucose absorbed/100 g/hr.

† Sample too small for statistical analysis.

TABLE II. Mean Rates of Weight Gain for Control and HO Rats.

	Rate of gain, g/day	Range, g/day	S.E.
Control	2.87	1.9-3.8	±.215
HO	3.77*	1.9-5.5	±.383

* 36% increase over controls, significant at 5% level.

intraperitoneally, the abdomen opened, and ligatures placed around the ascending part of the third portion of the duodenum and the lowest ileum. A measured volume (3 cc/100 g) of isotonic glucose was introduced into the ligated intestinal loop by multiple injections. After a 25-minute absorption period the rats were sacrificed. Residual glucose was estimated by the Somogyi-Nelson colorimetric method(5). The absorbed glucose was expressed as the glucose coefficient of absorption (GCA) = mg glucose absorbed/100 g/hr. Statistical analysis utilized the method of Student's t.

Results. The absorption data are presented in Table I. The GCA of 114.0 for the controls is close to the value of 116.6 obtained in earlier work with this technic(4). The GCA of 107.4 for the HO rats does not differ significantly from that obtained for the controls ($p = .5$-$.6$).

Thyroidectomy depressed the GCA of unlesioned rats to 75.2, while thyroxine treatment elevated it to 191.6. These changes are of about the order reported by Althausen and Stockholm(6), indicating the method to be as sensitive as the Cori technic (used by these workers) in demonstrating alterations of the GCA in either direction.

The GCA of 173.5 for the thyroxinized HO rats indicates that the glucose absorptive capacity of these animals responds to changes in thyroid status.

In Table II are the mean rates of weight gain for rats from the control and HO groups. The obese rats had thick deposits of subcutaneous fat (particularly prominent in the thoracic region, where little or no fat is deposited in the normal rat) and massive retroperitoneal deposits of adipose tissue. Heavy deposits of fat were found in all obese animals at these sites, as well as in the mesentery, omentum, and pericardial region. (HO thyroxinized rats lost much of this excessive adiposity during the brief period of hyperthyroidism). Even though some obese animals gained less weight than some controls, there could be no doubt of their excessive corpulence.

Discussion. Our failure to find a difference between the GCA for unoperated rats and that for animals with distinct hypothalamic obesity might be interpreted in 2 ways: either that the use of anesthesia in these experiments obscured the existence of an altered rate of intestinal absorption of glucose in HO rats, or that HO may exist without alterations in the rate of glucose absorption by the intestine.

Anesthesia appears to lower the rate of intestinal absorption of all substances thus far examined in a uniform, nonspecific manner (7,8). Experimental alterations of glucose absorption under anesthesia correlate well with those reported in the unanesthetized animal(6,9). In the present experiment we have shown that nembutal does not prevent the rise in GCA resulting from thyroxinization of both unlesioned and HO rats. We therefore believe that the first interpretation of our findings would have little validity, and that HO can develop without an increase of GCA above that found in controls.

This finding is compatible with the concept that HO primarily owes its origin to hyperphagia(1). Although many other metabolic processes remain to be examined in HO animals, we are thus far unaware of any findings which refute the opinion advanced by Brobeck (10) that those metabolic derangements which have been found in such animals are readily

explained as secondary phenomena resulting from the hyperphagia.

On the other hand, the development of unquestionable obesity in some of our lesioned rats which did not gain weight more rapidly than the controls suggests that they deposited fat at the expense of other body constituents. Apparently a slowing or cessation of growth can obscure the significance of weight gain as a criterion of obesity. Cessation or diminution of growth together with obesity was observed by Hetherington and Ranson in hypophysectomized HO rats(11) and by Scow(12) in force-fed thyroidectomized rats. In both of these cases a combination of growth hormone deficiency (resulting in negative N balance) and excessive food intake could explain the phenomenon(12). The same explanation might well hold for our HO rats which did not exhibit an excessive weight gain. Support for this concept comes from the observation(13) that the anterior pituitaries of many of the lesioned rats show a striking degranulation of the acidophiles, similar to that found after thyroidectomy. This suggests that the secretion of growth hormone by these cells might be severely disturbed by hypothalamic lesions which also produce obesity.

Summary. 1. Hypothalamic obesity (HO) was produced in rats by means of electrolytic lesions. Glucose intestinal absorption determinations yielded the following glucose coefficients of absorption (GCA): Control, 114.0; HO, 107.4; HO thyroxinized, 173.5; thyroxinized, 191.6; thyroidectomized, 75.2. These results indicate that the intestinal absorption of glucose need not differ from control values in order for obesity to occur. 2. Thyroxine elevated the GCA of HO rats as well as that of unlesioned rats. Unlesioned thyroidectomized and thyroxinized rats served as method controls. 3. Observations on HO rats which did not exhibit excessive weight gain suggest that hypothalamic lesions may interfere with the secretion of growth hormone by the pituitary.

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Comparison of Effects of Various Brands of ACTH on Normal Subjects.* (19894)

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The purpose of this study was to compare the effects of several types of commercial brands of ACTH in their ability to produce a reduction in circulating eosinophils and to increase the urinary excretion of 17 ketosteroids in human subjects. Additional studies were aimed at depleting the ascorbic acid contents in the adrenal glands of hypophysectomized rats by means of injections of the same lots of ACTH as were used in the human subjects. Commercial preparations of ACTH have their dosages based on the rela-

* I wish gratefully to acknowledge the advice of Professor Paul Starr and the statistical treatment of Dr. Fred Moore.

TABLE I. Change in Blood Eosinophil Levels Before and After Administration of the Different Brands of ACTH and Normal Saline, as Related to Specific Brands and the Individual Subjects.

Drugs		Subjects J.B.	R.C.	L.S.	L.M.	M.M.	Avg % decrease/brand
Brand 1	No. of eosinophils at 0 and 4 hr →	218– 73	113– 48	138– 44	144– 50	350– 52	
	% decrease →	75%	57%	68%	65%	85%	70
2		116– 58	149– 94	84– 20	166– 78	367– 98	
		50%	37%	76%	53%	73%	57.8
3		147– 95	125– 59	84– 89	95– 33	56– 25	
		35%	53%	+6%	65%	55%	40.4
4		170– 53	111– 33	127– 43	211– 78	144– 45	
		69%	70%	66%	63%	69%	67.4
5		135– 61	175–117	92– 73	200– 94	102– 42	
		55%	33%	21%	53%	59%	44.2
Norm. saline		259–183	272–218	116–100	170–128	232–130	
		29%	20%	14%	25%	44%	26.4
Avg % decrease/subject		56.8%	50%	45%	59.9%	68.2%	

Columns of percentages at far right and at extreme bottom are the avg percentage changes in eosinophils as related to individual brands and subjects respectively. Eosinophils are expressed in total No./cc.

tive potency of their products as determined by the biological assay of Sayers, Sayers and Woodbury(1). This assay depends on the ability of intravenous ACTH to deplete the ascorbic acid content present in the adrenal glands of hypophysectomized rats. Greenspan and coworkers(2) however have noted the lack of correlation of ACTH potency as determined by the Sayers method and other adrenal measurement tests such as the maintenance test. Reinhardt and coworkers(3) have concluded that the ascorbic acid depletion test is but one effect, and as such, is an incomplete measure of the various possible responses to ACTH administration in experimental animals.

Methods. For purposes of this study, young male medical students were selected. The lots of ACTH were prepared by E. R. Squibb and Son, United Laboratories, Ltd., Wilson Laboratories, National Drug Co., and Armour Co. All ACTH preparations were made into solution just prior to use unless originally prepared in a liquid state. At intervals of no less than one week, intramuscular injections of one of the various lots of ACTH or normal saline were given to each subject. At no time were the subjects aware of the type of injection received, and eventually they all were given each lot of ACTH and normal saline. The dosage of ACTH was equivalent to 25 mg and that of normal saline, 1 cc. Eight hours prior to testing oral intake was restricted to either water or orange juice. A 2 hour urine specimen was obtained from 6 a.m. to 8 a.m., just before receiving the injection. At 8 a.m., capillary blood specimens were also obtained. Beginning 1½ hours after the injection of the unknown, another 2½-hour urine specimen was collected between 10:30 a.m. and 12 noon. At this last hour, capillary blood was again drawn. The tests performed with these collections were the 4-hour Thorn Eosinophil Test(4) and the measurement of urinary 17 ketosteroids as determined by the method of Talbot and coworkers(5). Additional studies with hypophysectomized rats were used employing the biological assay of Sayers to evaluate the ability of these lots of ACTH and the Armour standard (La-1 A) to deplete the ascorbic acid content in their adrenal glands.† A dosage of 2 gamma of ACTH/100 g rat weight was used.

Results. An average decrease in blood eosinophils of 57% or greater was obtained with 3 of the 6 products tested in the human subjects (Table I). Brands No. 1, 2, and 4 were associated with decreases of 70%, 67%, and 57% respectively. Brands No. 3 and 5, with average decreases of 40% and 44%,

† Sayers' bioassay data are the results obtained from the United Laboratories, Ltd., Pasadena, Calif., through the courtesy of Mr. Richard Bruner.

TABLE II. Changes in mg of 17 Ketosteroids/Hr Before and After Administration of Different Brands of ACTH and Normal Saline, as Related to Specific Brands and Individuals.

Drugs		Subjects J.B.	R.C.	L.S.	L.M.	M.M.	Avg change/brand
Brand 1	mg of 17 ketosteroids →	.895–1.788	1.300–1.102	.846–1.156	.875–1.338	.463–.690	.361+
	Change in levels→	.893 ↑	.198 ↓	.310 ↑	.463 ↑	.227 ↑	
2		.748– .940	.849– .783	.895–1.305	.714– .937	.513–.692	.188+
		.192 ↑	.066 ↓	.410 ↑	.223 ↑	.179 ↑	
3		.719– .944	.702– .860	1.019– .952	.583– .556	.484–.645	.090+
		.225 ↑	.158 ↑	.067 ↓	.027 ↓	.161 ↑	
4		.749–1.727	.669– .938	.710–1.250	.442– .702	.513–.711	.449+
		.978 ↑	.269 ↑	.540 ↑	.260 ↑	.198 ↑	
5		.700– .929	.895– .769	.746– .778	.648–1.065	.527–.967	.208+
		.229 ↑	.126 ↓	.032 ↑	.417 ↑	.440 ↑	
Norm. saline		.540– .951	.832– .779	.780–1.003	.466– .596	.542–.498	.133+
		.411 ↑	.053 ↓	.223 ↑	.130 ↑	.044 ↓	
	Avg change/subject	.543 ↑	.001 ↑	.244 ↑	.329 ↑	.241 ↑	

Avg change in quantitative amounts is recorded in columns to extreme right and at bottom of the chart, and reflects changes among individual brands and subjects respectively. Direction of arrow indicates either a rise or fall in the change of levels.

had 3 responses which were greater than 50%, but failed to elicit greater decreases in other tests. The average decrease with normal saline was 26% with variations from 14 to 44%. All subjects demonstrated an average decrease in eosinophils of at least 45% or more with ACTH. The responses to normal saline were much less pronounced. Subject L.S., having the least response with ACTH, showed the least change with normal saline whereas subject M.M., showing the best response, almost demonstrated an ACTH-like response with normal saline.

The individual 17 ketosteroid responses as related to the specific lots of ACTH and normal saline are summarized in Table II. All the drugs, including normal saline, were associated with some varying degree of increase in levels in the urine specimens obtained after the injections were made. In one instance, the changes with normal saline exceeded those of ACTH. Considering the total amounts, the greatest differences among the ACTH preparations were within close range of each other. Although the individual subjects tended to have initial levels within a close range, they varied widely in their responses. Subject J.B. demonstrated the greatest increases, but the response to normal saline was also relatively excessive. The 17 ketosteroid levels in subjects L.M. and M.M. showed the greatest differences in responses to ACTH and normal saline. Subject R.C. demonstrated only slight changes with all products.

The results of the comparison of the ascorbic acid depletion tests as caused by the different lots of ACTH and the Armour standard are summarized in Fig. 1. All lots of ACTH possessed a varying degree of potency, but always that below the standard. Those lots associated with the most intense depletion were Brands No. 2, 3, and 5. Brands No. 1 and 4 when tested in humans showed the most marked responses with 17 ketosteroid increases and eosinopenia, but in the animal test they showed the least.

Summary. An analysis of the accumulated results indicates the following: 1. There is a statistically significant difference between ACTH and normal saline as related to induced eosinopenia. Furthermore, differences among brands are apparent with Brands No. 1, 2, and 4 producing the more profound effects. 2. 17-Ketosteroid levels are slightly increased by all the brands and also by normal saline. Although Pincus(6) has been able to produce sharp hourly rises in urinary 17 ketosteroids under situations of induced stress in man, it seems that an intramuscular injection of 25 mg of ACTH is not capable of duplicating this, at least from urine specimens collected at these

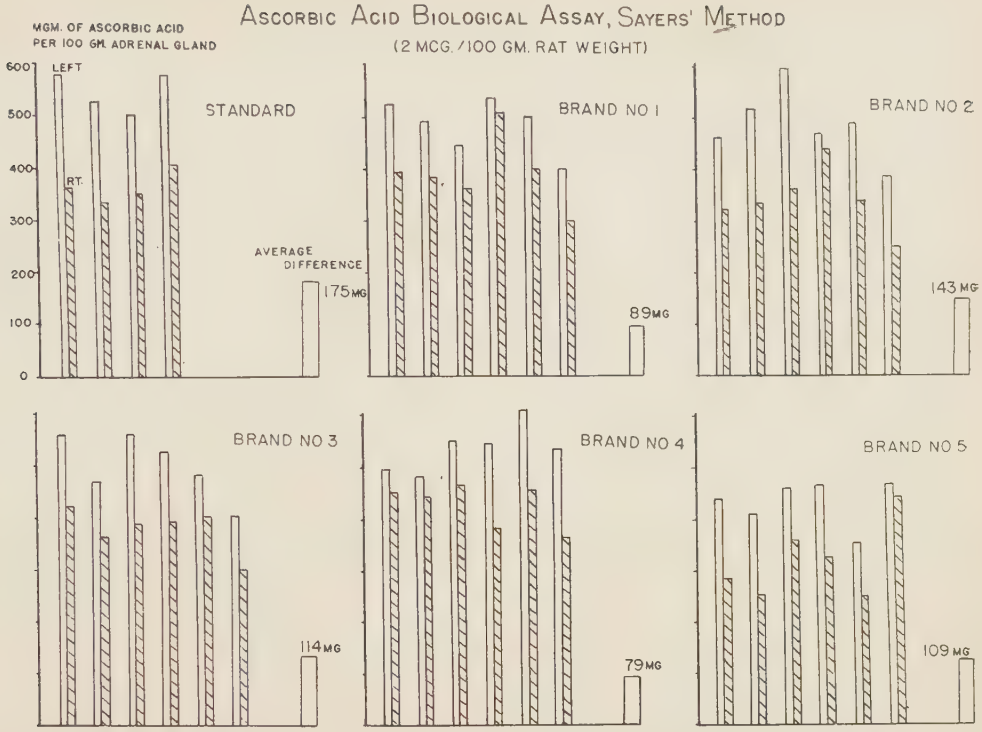

FIG. 1. Different brands of ACTH are compared to the known Armour standard by the use of the Sayers' ascorbic acid biological assay. The plain and striped paired columns indicate mg of ascorbic acid/100 g adrenal gland before and after administration of ACTH. Columns are labeled right and left, indicative of the respective glands, and depleted levels in the right adrenal glands occurred following ACTH. The other column at extreme right in each grouping shows avg differences in ascorbic acid levels of the paired adrenal glands.

time intervals. 3. There is no direct correlation among the three tests performed, but there seems to be an inverse relationship between the biological assay in rats and the tests in humans. 4. Because of this variation between animal and human responses, and the results of other investigative work as referred to above, it seems to be questionable whether the present ascorbic acid depletion animal bioassay is a completely satisfactory method of determining the degree of activity of ACTH in man.

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Effect of Adrenalectomy on Rate of Metabolism of Histamine in the Mouse.* (19895)

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(Introduced by Alvin F. Coburn.)
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Rose and Browne(1) have shown that adrenalectomized rats have a markedly decreased ability to inactivate injected histamine as compared to intact animals. The levels of histamine employed were necessarily very large, 24 μg per g of body weight. This change could be restored essentially to normal by administration of adrenocortical extract(2).

Because of the association of histamine with certain inflammatory states, and the anti-inflammatory activity of cortisone and ACTH, the implications of these findings are of considerable importance. The availability of radioactive histamine made it possible to repeat these experiments using much lower histamine concentrations. The mouse was chosen as the experimental animal so that the entire carcass could be analyzed for histamine.

Materials and methods. The synthesis of histamine, labeled in the 2-position of the imidazole ring, has been published(3). Male Swiss albino mice weighing approximately 20 g were adrenalectomized† or sham-operated 3 days before injection. In one experiment mice were injected intraperitoneally with 0.05 μg C^{14}-histamine‡ per g of body weight (Table I); in a second experiment the level was 1.0 μg C^{14}-histamine per g of body weight. (Table II). At various intervals of time after injection the mice were killed; the urinary bladders were removed, excepting mice killed less than 10 minutes after injection; the carcasses were immediately frozen in dry ice-acetone. The mice were skinned and each homogenized (Waring blendor) in water containing 40 mg carrier histamine; the skins were then added and homogenization continued. Protein was precipitated with trichloroacetic acid and histamine extracted from the filtrate and isolated as the dipricrate in a manner similar to that previously described (4). The histamine dipicrate was readily

TABLE I. % of C^{14}-Histamine in Mouse Carcass at Various Time Intervals Following Intraperitoneal Injection. .05 μg histamine/g body wt.

	Min.										
	3	5	7	10	12	15	20	30	60	90	120
Adrenalectomized	73	65	62	35	42	40	19	13	4	4	2.5
Sham-operated	69	67	34	32	38	27	24	10	4.5	3.4	1.8

TABLE II. % of C^{14}-Histamine in Mouse Carcasses 15 and 30 Min. Following Intraperitoneal Injection. 1 μg histamine/g body wt.

	Adrenalectomized	Sham-operated
15 min.	42	43
	33	32
	50	31
30 min.	22	20
	25	27
	35	47
	37	48

* Supported by a contract with the U. S. Atomic Energy Commission.

† The authors are indebted to Dr. Georges Ungar and Miss E. Irene Pentz for demonstrating the proper technic of adrenalectomy.

‡ Histamine solutions were in 0.004 N hydrochloric acid. The previously suggested procedure(5) of neutralization with sodium bicarbonate is not recommended if an appreciable time elapses before use; significant deterioration can occur in very dilute solutions under these conditions.

‖ In many previous assays using this procedure we have found that minute amounts of C^{14}-histamine can be readily separated from very large excesses of histidine, histidine metabolites, and histamine metabolites, all of comparable radioactivity. In some cases the histamine dipicrate was recrystallized many times, using Norit, and often employing two different solvents alternately. The radioactivity was invariably constant after the second crystallization. To add further assurance that the radio-activity being measured was due to histamine and not a contaminant, histamine dipicrate samples were converted to the free base. In each case, after sublimation, the free histamine showed the calculated increase in specific activity.

purified to constant radioactivity by recrystallization from hot water using Norit.‖ The original histamine solution was assayed by adding the same amount used for injecting the mice to the usual amount of carrier (40 mg) then preparing and counting the dipicrate. For example 1.00 μg C^{14}-histamine added to 40 mg carrier, after conversion to the dipicrate, produced 500 counts per minute when counted at infinite thickness and corrected for background only. When 1.00 μg C^{14}-histamine was injected into a mouse the isolated histamine dipicrate produced 200 counts per minute at infinite thickness and corrected for background only. Therefore, the percentage of histamine remaining in the entire mouse is $200/500 \times 100 = 40\%$. All counts were made on 4.5 square cm plates in flow counters with background about 20 c.p.m.

Results. The data of Tables I and II show that although there are often marked differences between individual mice treated identically, there is no significant difference in the rate of histamine destruction between adrenalectomized and sham-operated mice. This discrepancy with the findings of Rose and Browne may be due to differences in histamine concentrations administered, species differences, or variations in experimental procedure. In any event the results suggest that the participation of the adrenal cortex in histamine inactivation under physiological conditions is questionable and that the matter requires further clarification.

Summary. No significant difference was found between adrenalectomized and sham-operated mice in the ability to destroy minute quantities of injected C^{14}-histamine.

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Effect of Epidermal Damage upon Serum Polysaccharides. (19896)

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(Introduced by A. A. Hellbaum.)

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Elevations of serum polysaccharides have previously been reported by Shetlar, *et al.*(1), to occur in dogs following production of sterile turpentine abscesses, of bacterial abscesses and of talc granulomas, the injection of turpentine intrapleurally, and experimental surgery. The cause and mechanism of these elevations is of considerable interest. Seibert *et al.*(2), have suggested that the elevation noted in malignancies, tuberculosis, and other pathological conditions is related to tissue destruction. Shetlar *et al.*(1) have suggested that this phenomenon may just as logically be correlated with tissue proliferation and repair. Since a number of carbohydrate rich protein fractions occur in serum, it also seems likely that they may vary differentially. Shetlar *et al.*(3) using a sodium sulfate fractionation procedure, noted that the distribution of polysaccharide among different serum protein fractions varied in different diseases. In the course of a study of treatment of thermal injury in dogs the opportunity to study this problem further presented itself. If the source of the serum polysaccharides, which rise after injury, is the injured tissue itself, one would expect the lymph polysaccharides to rise after injury at a more rapid rate than those of the serum. Consequently, the experiment was designed to allow polysaccharide studies of lymph samples from dogs at various times after burning.

Methods. In this study nonglucosamine

* Contribution No. 25 from the Oklahoma Medical Research Foundation.

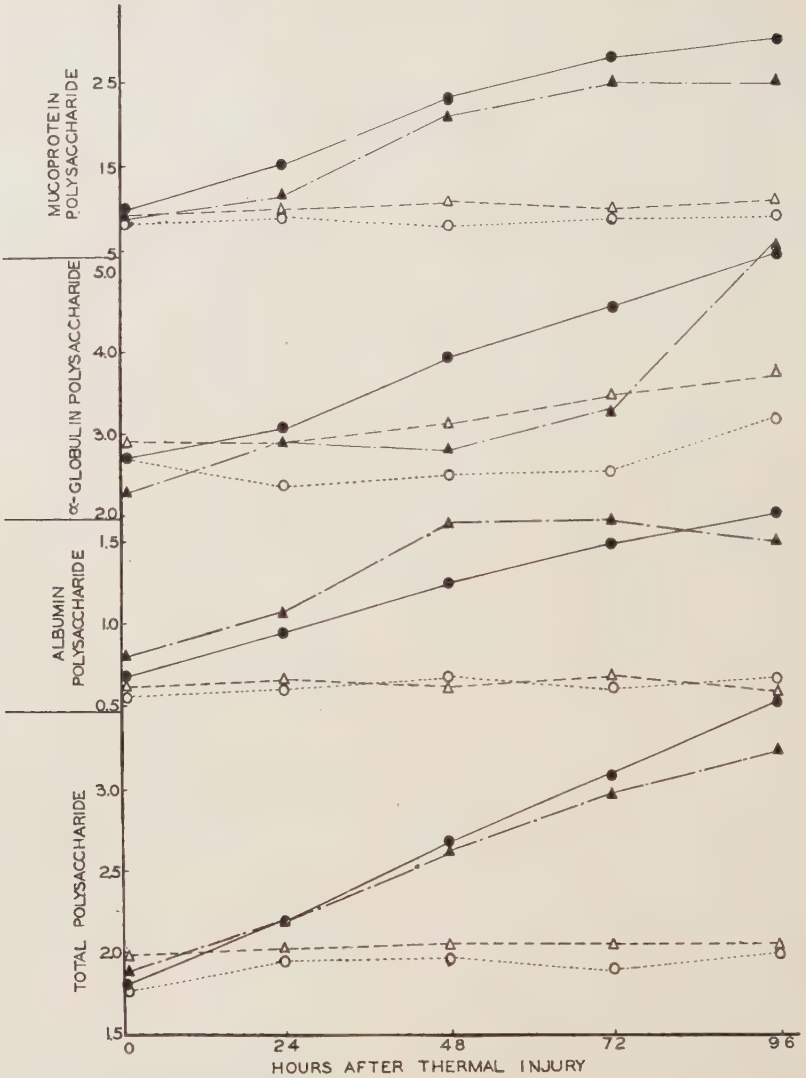

FIG. 1. Effect of epidermal thermal injury on nonglucosamine polysaccharides of serum protein. The different groups are denoted graphically, (1) untreated injured ●——●; (2) untreated uninjured ○------○; (3) ACTH treated injured ▲– – – –▲; (4) ACTH treated uninjured △— — —△. Total polysaccharide is denoted as % of the serum protein, "albumin" polysaccharide as % of the albumin protein, "α-globulin" polysaccharide as % of α-globulin, and mucoprotein polysaccharide as mg of hexose per 100 ml of serum.

polysaccharide was determined by the tryptophan method(4). Serum and lymph were fractionated as previously described(3) by precipitation with 21.6% and 26.0% sodium sulfate. As indicated by Gutman(5), the protein not precipitated by 26% sodium sulfate was assumed to be largely albumin and shall be designated in this paper as the albumin fraction, while that not precipitated by 21.6% was assumed to be combined albumin and pseudoglobulin (α-globulin). Figures obtained by subtracting the results of analyses of the 26% from the 21.6% sodium sulfate fraction were considered to be representative of pseudoglobulin and shall be designated hereafter in this paper as the α-globulin fraction. Mucoprotein polysaccharide was determined by the tryptophan method after isolation of the mucoprotein fraction by the method of Winzler and Smyth(6). Mongrel dogs weighing from

TABLE I. Comparative Changes of Nonglucosamine Polysaccharides of Lymph and Serum Following Experimental Epidermal Thermal Injury.

Hr after burning	Total protein[1]		α-globulin[2]		Albumin[3]		Mucoprotein[4]	
	Lymph	Serum	Lymph	Serum	Lymph	Serum	Lymph	Serum
0	1.51	1.76	2.56	2.37	.84	.56	6	8
0	1.98	2.03	2.44	3.22	1.51	.66	6	8
24	1.40	2.12	3.46	2.14	.98	.87	7	10
48	1.76		3.34		1.45		20	
72	2.84	2.80	6.66	4.30	1.47	1.66	22	26
72	2.52	2.48	6.55	4.05	1.17	1.07	12	14
72	2.58	3.19	5.19	5.90	1.85	1.64	39	34
96	2.80	3.69	5.06	3.69	1.71	1.25	22	25
96	2.64	3.58	4.75	5.26	2.37	1.91	28	33

Expressed as % of [1] the total serum protein, [2] α-globulin protein, [3] albumin protein, and [4] as mg/100 ml of serum.

10 to 20 kg were selected. After shaving the skin and under intravenous nembutal anesthesia, second and third degree burns were induced with electric cauterization. Burns were made over the medial and lateral surfaces of both legs, flanks and abdomen to the costal margin. No dressings were applied other than petroleum jelly. Under nembutal anesthesia a skin incision was made over the medial border of the lower one-half of the left sternocleidomastoid muscle. The left jugular vein was exposed and dissected free to its junction with the subclavian vein. The thoracic duct was identified at its termination at the venous angle. The external jugular vein, the innominate vein and all their tributaries were ligated above and below the venous angle. The external jugular vein was then cannulated with a polyethelene tube and the thoracic duct lymph collected in a small beaker and allowed to clot. The lymph sera were later separated by centrifugation.

Results. Changes of serum polysaccharides. The results of experiments involving 5 untreated injured dogs, 2 injured dogs treated with adrenocorticotropic hormone, 2 untreated normal controls, and 2 controls injected with adrenocorticotropic hormone are presented graphically in Fig. 1. The total nonglucosamine serum polysaccharide was divided by the serum protein. The average results for each day after thermal injury was compared statistically with the data obtained before injury. The term "significantly" is used here to denote differences significant at the 1% level. A noticeable, but not statistically significant, elevation occurred in every case within 24 hours after burning. At 48 hours the total nonglucosamine serum polysaccharide was significantly elevated. Dogs treated with ACTH were not noticeably different from the untreated. The polysaccharide of the α-globulin fraction (Fig. 1) was also noticeably, but not significantly elevated within 24 hours after injury and significantly elevated at 48 hours. Elevation of this factor in the dogs treated with ACTH was apparently repressed for the first 72 hours. However, after this time, the level became as high as in the untreated dogs. The polysaccharide associated with the albumin fraction, as isolated by the precipitation of globulin with 26.0% sodium sulfate, was found to be significantly elevated 24 hours after injury and continued to rise until death as shown in Fig. 1. By this method of fractionation, the mucoprotein is found in the albumin fraction(7). The elevation of the polysaccharide of the albumin fraction was entirely explainable by the rise which occurred in the mucoprotein polysaccharide depicted in Fig. 1.

Changes in lymph polysaccharides. Due to the necessity of sacrificing the animal in order to obtain the lymph, serial determinations of lymph polysaccharides in the same animal were not possible. As considerable variation between animals was noted, care must be taken in interpreting the data obtained on lymph. A study involving more animals is indicated. However, the data, summarized in Table I, indicate that the total nonglucosamine polysaccharide increases in lymph after burning. Much of this increase is due to the increase of polysaccharide in the fraction precipitated by

26% but not by 21.6% sodium sulfate (α-globulin fraction). The polysaccharide found in the albumin fraction also appears to rise but the elevation is not so striking. The polysaccharide associated with the mucoprotein fraction is consistently elevated in the lymph following burns. It is noteworthy that the figures for each lymph sample and its corresponding serum sample are practically the same. It appears likely that mucoprotein passes freely back and forth between tissue fluids and blood.

Discussion. Thermal injury to experimental animals results in an elevation of serum polysaccharides. This response is probably no different than that previously noted in other injuries(1). As the animals in this study were either sacrificed or died as a result of the injury, data as to the maximum elevation were not obtained. In contrast to cancer patients or pregnant women, the polysaccharide of the albumin fraction after correction for mucoprotein polysaccharide was not elevated in these animals. It appears that the elevation of serum polysaccharide in burned animals is due largely to increases of the mucoprotein fraction and of a polysaccharide rich component which occurs in the α-globulin fraction as isolated by sodium sulfate fractionation. After completion of this work Keyser(8) reported a study on patients which included several burned cases, one of which was followed for some time serially. He estimated total serum nonglucosamine polysaccharide and mucoprotein polysaccharide. He reports an elevation of both total and mucoprotein polysaccharide. The rise of the total polysaccharide is not completely due to the mucoprotein elevation. Apparently in humans, as in canines, an elevation of both mucoprotein and another carbohydrate rich fraction occurs. Treatment of burned animals with ACTH may influence the elevation of the polysaccharide of the α-globulin fraction, but not that of the mucoprotein. Since the differences between the polysaccharide levels of the lymph and serum were not conclusive, the question as to whether the elevation of serum polysaccharide is derived from the injured tissue requires further study.

Summary and conclusions. Epidermal thermal injury in dogs resulted in an elevation of total polysaccharide in both lymph and serum and of the polysaccharide of both the albumin and α-globulin (pseudoglobulin) fractions isolated by salt precipitation methods. The elevation of the polysaccharide of the albumin fraction was shown to be due to an elevation of the mucoprotein polysaccharide. The mucoprotein content of the lymph and serum from the same animal were nearly the same indicating that this component may pass easily between tissue fluids and blood plasma. Preliminary studies of the effects of adrenocorticotropic hormone on animals with epidermal thermal injuries indicate that this substance has no effect on the elevation of mucoprotein, but may repress the elevation of α-globulin polysaccharide.

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A New Host-Virus System.* (19897)

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The inability to manipulate animal cells constitutes one of the serious limitations in the investigation of the mechanism of viral propagation. Animal tissues which are susceptible to viral infection exist as organized aggregates of several types of cells. The production of a simultaneous uniform infection, the determination of cellular viability, or even the estimation of the number of cells involved most often is impossible. Ascitic tumor cells of a homogeneous type can be obtained, and when properly cultured in the mouse peritoneal cavity, they will grow as discrete cells which can be counted in a hemocytometer (1,2). Unlike most adult animal cells, their viability can be tested by implantation to a susceptible host. Because of these desirable properties, ascitic tumor cells were considered as a possible component of an experimental host-virus system.

In this preliminary report the adaptation of an influenza virus to culture in an ascitic form of a mouse tumor is described.

Materials and methods. Tissue. The Ehrlich mouse carcinoma used in these experiments was obtained in ascitic form from Dr. G. A. LePage. In this laboratory it has undergone 45 passages in Swiss white mice (Webster strain). For each passage ascitic fluid was removed from the peritoneal cavity of mice which had been inoculated with tumor cells 7 days previously. After counting the cells in a hemocytometer, appropriate dilutions were prepared in saline and mice were inoculated intraperitoneally with approximately 20 million cells. *Virus.* The WS strain of Type A influenza virus was chosen for these studies. This virus was maintained for several years by continuous passage in tissue culture and since has undergone 102 passages in mouse brain (3). It will multiply both in the brain and lungs of mice. *Virus titer.* The amount of virus was estimated by determining the infectious titer for eggs. Tenfold serial dilutions of the virus were prepared in broth and 4 eggs were inoculated with 0.1 ml of each dilution. After 3 days of incubation at 37°C, samples of allantoic fluid were removed from each egg. These fluids were tested for virus by the addition of chicken red blood cells. The 50% infectious titer was calculated using the method of Reed and Muench (4).

Results. Adaptation of influenza virus to the Ehrlich ascites tumor. Five mice, bearing 5-day-old ascitic tumors, were inoculated intraperitoneally with 0.2 ml of a 10% suspension of infected mouse brain which had an infectious viral titer for eggs of $10^{-5.0}$. Three days later ascitic fluid and cells were removed from these mice. One portion of the fluid was titered in eggs for virus content, and a second portion was used to infect a new group of mice which bore ascitic tumors. At 3-day intervals the process of serial passage was repeated. After each passage, the egg infectious titer of the ascitic fluid was found to be lower than the previous one until by the third passage no infectivity for eggs was demonstrable (Fig. 1). This material then was used to inoculate mice intracerebrally. When the brains from these mice were harvested, they showed an infectivity titer for eggs of $10^{-5.5}$. A 10% suspension of the brain was prepared and used to initiate a new series of serial passages in the ascitic tumors. By the third passage of this latter series, the infectivity titer had again decreased to $10^{-1.5}$. Thereafter, the titer of each successive passage increased until titers as high as $10^{-5.0}$ to $10^{-6.0}$ were steadily attained (Fig. 1). The virus has now undergone 38 serial passages in ascitic tumors.

Growth characteristics of influenza virus in ascitic culture. Mice bearing ascitic tumors were inoculated with 0.2 ml of a suspension

* Aided by a grant from the National Foundation for Infantile Paralysis.

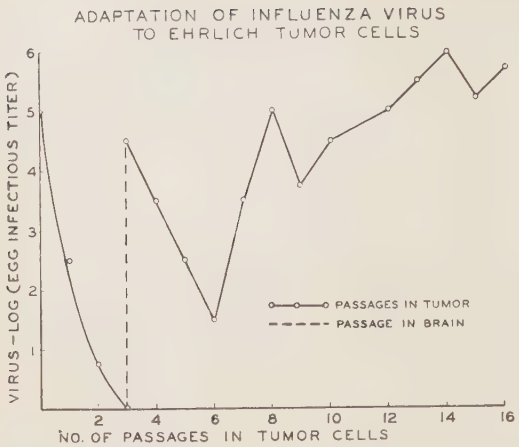

FIG. 1. Duration of each passage was 3 or 4 days. The virus was passed undiluted during the first 7 passages and thereafter at a dilution of 10^{-1}. All estimations of the amount of virus are based on infectious titers for eggs.

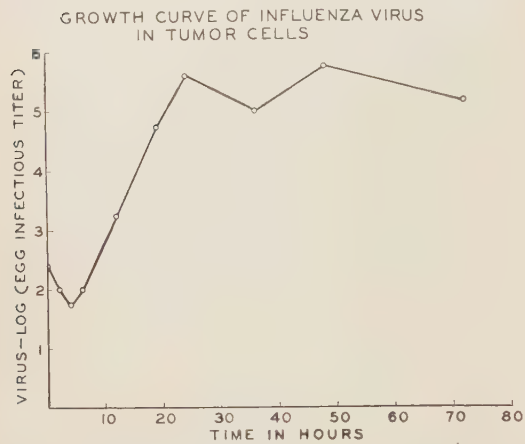

FIG. 2. Each mouse received .2 ml of an inoculum which had a titer of $10^{-5.4}$. This virus had undergone 33 previous passages in ascitic cells. The virus titers recorded are infectious titers for eggs.

of the adapted virus which had an infectivity titer for eggs of $10^{-4.7}$. Five mice were sacrificed immediately, and the ascitic cells were found to have a titer of virus of $10^{-2.5}$. At appropriate intervals thereafter groups of mice containing 5 animals were sacrificed, and the ascitic cells removed. The titers of influenza virus of pooled samples of the tumor material were determined in eggs. By 4 hours after inoculation, the titer of the peritoneal fluid had decreased further to $10^{-1.7}$. An increase in virus titer was first detectable at 6 hours and by 12 hours was $10^{-3.5}$. The maximum titer ($10^{-5.5}$ to $10^{-6.0}$) was reached by 24 hours and remained relatively constant through 72 hours (Fig. 2).

Effect of influenza virus on Ehrlich tumor cells. Tumor cells were removed from the peritoneal cavity of mice which had been inoculated 2 days before with influenza virus. Examination under phase microscopy revealed no difference in gross appearance from the usual tumor cells. However, repeated attempts to initiate a new ascitic culture with these cells were without success. When an equal number of tumor cells uninfluenced by virus was employed, tumors were induced in all the animals. Thus, it appears that the infection of these cells is accompanied by a loss in viability of the host cell.

In view of this finding, it was of interest to determine the effect of the virus on the course of the development of an ascitic tumor culture which had been securely established prior to the introduction of the virus. One group of mice which had been implanted with tumor cells 5 days before was inoculated with 0.2 ml of a virus preparation having a titer of $10^{-4.5}$. When this group was compared with a control containing no virus, it was observed that in 2 days the amount of ascitic fluid was markedly reduced (controls containing 3 ml, while the virus treated contained 0.5 ml per mouse). Further, the ascitic cells in the infected animals had begun to aggregate and were found in the peritoneal cavity in large clumps. By the 7th day after the introduction of the virus, the control animals were greatly distended, while those with the virus appeared normal in size (Fig. 3). When the latter animals were sacrificed, none or only small aggregates of cells could be found in the peritoneal cavity. Thus, accompanying the inhibition of the multiplication of the tumor cells is an extensive regression of the tumor.

In several experiments a large number of mice died after the regression of the tumor presumably from the invasion of the virus. However, in other instances a considerable number of mice continued to survive until sacrificed 6 to 7 weeks later. A systematic examination of the variables which might produce these inconsistent results has not been completed. However, it will be noted that

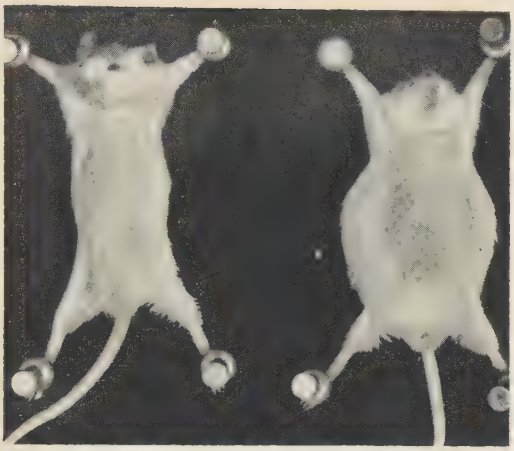

FIG. 3. Effect of virus on development of ascites tumor. Each mouse was inoculated with 20×10^6 Ehrlich tumor cells. Five days later the mouse on the left received intraper. .2 ml of a 10% suspension of virus which had undergone 15 passages in ascitic tumor cells. The photographs were taken 16 days after the viral inoculation.

the intraperitoneal inoculation of normal mice with this adapted strain of virus is never accompanied by fatalities.

Discussion. The multiplication of an adapted strain of influenza virus in an ascitic mouse tumor can be demonstrated by continuous serial passage or by following the growth curve during a single passage (Fig. 1 and 2). It appears that this viral multiplication in the tumor tissue is accompanied by a loss of viability of the host cells and a gross recession or destruction of the tumor itself (Fig. 3). As yet, experiments of long duration which would be necessary to evaluate thoroughly the use of this phenomenon in tumor therapy have not been completed.

This new experimental host-virus system should be of particular value in the study of the mechanism of viral infection, since it is possible to produce an infection simultaneously in a large number of cells of one type. Further, infected cells may be observed individually. While many new and interesting types of experiments can be performed with this system, accurate quantitative experimentation can best be done in tissue culture. Attempts to grow this virus in ascitic cells which are surviving in Warburg flasks are now in progress(5). How widely applicable this technic will be with regard to types of viruses and tumors also remains to be determined. However, the experiences of other workers with solid tumors may be indicative of the limitations to be encountered here(6,7).

Summary. A new host-virus system is described. The WS strain of Type A influenza virus was adapted to culture in the Ehrlich ascitic carcinoma of mice. This adapted strain of virus has been maintained through 38 serial passages in this tissue. The growth characteristics during a single passage have been described. Associated with the multiplication of this virus is a loss of viability of the host cell. The effect of this phenomenon on the course of development of an established ascitic tumor has been followed. The importance of this new host-virus system as a tool for the study of the mechanism of viral multiplication is discussed.

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Effects of Nutrition on Aspartic Acid Deaminase System of *Bacterium cadaveris.** (19898)

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The enzymic constitution of bacterial cells is known to be influenced by several factors, perhaps the most important of which are the nature of the growth medium, the conditions of cultivation (*i.e.*, pH, oxygen tension, temperature, etc.), and the methods of harvesting and preparing the cell suspensions. Whereas some of these factors have been reasonably well investigated(1) others have been neglected by the microbial physiologist.

The present paper is concerned with the effects of the concentration of medium constituents on the activity of the aspartic acid deaminase system.

Materials and methods. The organism studied was *Bacterium cadaveris* (Gale) cultured at 30-32°C for 16-18 hours in a medium composed of yeast extract, pepticase, and dipotassium acid phosphate in varying concentrations. The pH of the media was adjusted to 7. The cells were harvested by centrifugation and when washed were suspended in distilled water at 30-32°C, centrifuged for 15 minutes, and resuspended in distilled water at the same temperature to give a cell concentration of 0.5-1 mg of bacterial nitrogen per ml. The deamination experiments were performed at pH 7 in M/20 phosphate buffer at 37°C, using adequate controls without added aspartic acid. The reaction was terminated by the addition of 0.2 ml of 25% trichloroacetic acid, the tubes centrifuged, and an aliquot of the supernatant fluid removed for ammonia determination by Nesslerization. Color was measured in a Klett-Summerson photoelectric colorimeter using a blue filter (400-465 mμ).

Results. The effect of the concentration of the 3 constituents of the growth medium on the aspartic acid deaminase system of *Bact. cadaveris* was determined in the following manner. The basal medium (1% each of yeast extract and pepticase, and 0.5% K_2HPO_4) was varied by holding the concentration of two ingredients constant and using 0, 1, 3 and 5% of the other constituent in parallel experiments. The deaminase activity of the harvested cells was determined before washing and after 1, 2, and 3 successive washes in distilled water, in order to ascertain the effect of nutrition on the stability of the cell suspensions.

TABLE I. Effect of Concentration of Medium Constituents on Activity of Aspartic Acid Deaminase System of *Bacterium cadaveris.*

Constituent varied	%	μg NH_3-N produced (60 min.) No. of washings 0	1	2	3
Yeast extract	0	54.2	60.3	51	11.3
	1	53.7	52.2	43.2	29.6
	3	54.8	51.3	45.2	42.1
	5	62.6	60.3	60.9	54.8
Pepticase	0	29.9	28.7	16.8	5.8
	1	29.3	26.4	28.1	17.1
	3	28.7	24.9	25.2	25.2
	5	23.2	24.9	25.1	24.5
Phosphate	0	56	22	9.9	3.2
	1	52.5	59.6	34.8	23.1
	3	58	58.6	11.9	10.2
	5	51.9	45.8	11.9	8.1

The data for 3 representative experiments are given in Table I. It may be seen that the aspartic acid deaminase activity of the cell suspensions before washing had not been altered significantly by varying the concentration of any one of the constituents of the growth medium from 0-5%. The initial activities varied from one experimental batch of cells to another, hence the lower activity of the pepticase cells in the experiment cited (Table I) has no significance. However, examination of the results obtained after 2 and 3 washings reveals marked differences in the activity of the cells harvested from the several media. It is apparent that the activity of the cells after 3 washings in distilled water increased in direct proportion to the concentration of either yeast extract or pepticase in the growth medium. For phosphate

* This study was aided in part by a grant from the National Institutes of Health, Public Health Service.

the greatest activity appeared at a concentration of 1% with a definite decrease in activity below and above this concentration.

Inasmuch as there were no significant differences in initial activity regardless of the concentration of medium constituents employed, and since marked differences were noted after successive washings, it may be concluded that the nutritional status of the cells, insofar investigated, affected either the stability of the enzyme system or the permeability of the cell membrane. The experiments were carried out with and without the addition of cofactors (1 mg yeast extract per tube) with no significant difference in results, indicating that coenzyme concentration was not limiting in these studies. It is important to note that both the amount of growth and the final pH varied only slightly among the various media studied.

Discussion. The results given indicate that the initial enzymic activity (aspartate deaminase system) of the bacterial cells is not influenced by the variations in nutrition studied. Upon the application of a criterion not previously employed, namely enzyme activity after successive washings, it became apparent that the nutrition during growth does affect significantly the activity of this enzyme system. Further work is necessary in order to elucidate the mechanism of these effects. Two possible explanations may, however, be offered. These are altered enzyme stability or differences in cell membrane permeability. In the latter case the effect may be due to either lowered permeability to the substrate or increased permeability resulting in loss of enzyme from the cell.

These results are significant not only because they suggest a more careful appraisal of the effects of nutrition on an enzyme system under investigation, but also because they reemphasize the fact that the enzyme constitution of a particular bacterial suspension is that portion of its potential which is selected by the nature of the growth medium, the conditions of cultivation, and the methods of harvesting and preparing the cell suspensions.

It appears to us not at all improbable that more rigid attention to these factors will lead to the discovery of enzyme systems predicted but hitherto not described, and to the stability of systems at present difficult to study.

Summary. Data are presented to show that certain variations in nutrition, while not affecting the initial activity of an enzyme system, may influence markedly the stability of this system to successive washings.

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Prevention of Growth Hormone-Induced Diabetes in Hypophysectomized Dogs by Adrenocortical Steroids.* (19899)

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In an earlier investigation it was shown that growth hormone (Armour), in dosages of 1.0 mg/kg/day intramuscularly, completely abolished the insulin hypersensitivity of hypophysectomized dogs after several days administration, but always produced a concomitant diabetes and insulin resistance(1-3). In addition growth hormone usually produced toxic effects (anorexia, vomiting, lethargy, death). If growth hormone has a truly physiological role in carbohydrate metabolism, it was postulated that 1) administered growth hormone, in the proper dosages under proper conditions, should partially or totally abolish the insulin hypersensitivity of the hypophysectomized dog without producing diabetes and 2) could then be administered indefinitely without the

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appearance of any unphysiological effects. In an attempt to demonstrate such a physiological anti-insulin action without the production of diabetes, the dosages of the growth hormone regimens were reduced. When growth hormone was administered to hypophysectomized dogs in the smallest effective dose (0.02 mg/kg/day intramuscularly) that exerted noticeable anti-insulin effects, it was found that 50% of the dogs so treated still developed a diabetic-type of glucose tolerance(4).

Previous work had shown that either growth hormone(5,6) or C-11, 17-oxycorticosteroid (cortisone or hydrocortisone) regimens(7,8) could elevate the markedly depressed renal functions of hypophysectomized dogs only partially towards normal, but neither of these 2 hormonal factors could restore the renal functions completely to normal levels. In view of these findings, a combined adrenocortical steroid-growth hormone regimen was administered to hypophysectomized dogs to study the effect on renal functions,† and concurrently, observations were made on the changes effected in the carbohydrate metabolism of these animals. The present report is concerned with the effects of combined adrenocortical steroid-growth hormone regimens on the carbohydrate metabolism of hypophysectomized dogs.

Methods. A series of trained, unanesthetized female hypophysectomized dogs (53 to 222 days post-hypophysectomy) were used in this investigation. The technic of hypophysectomy(9),‡ after-care and feeding(3) of these animals are described elsewhere. All dogs had to exhibit a marked insulin hypersensitivity before they were chosen for this study. All experiments were done in the postabsorptive state. Insulin sensitivity was determined by the hypoglycemic response to 0.025 unit/kg insulin (Lilly)‡ administered intravenously (designated hereafter as the "test dose"); blood sugar changes were followed for 4 hours. In certain experiments a larger dose of insulin was used, 0.25 unit/kg intravenously, *i.e.*, 10× the test dose. The glucose tolerance was determined by the 4-hour blood sugar changes following a 10-minute intravenous infusion of 0.075 g/kg/min. glucose. The response to the hyperglycemic action of adrenaline was tested by a 5-minute intravenous infusion of adrenaline hydrochloride, 0.0035 mg/kg/min. The adrenocortical steroids, cortisone acetate or hydrocortisone (compound F) acetate‡ were given in daily dosages of 0.83 to 1.2 mg/kg intramuscularly in 2 divided doses (9 A.M., 5 P.M.) for periods of 17 to 38 days at which time growth hormone administration was begun, while the adrenocortical steroid regimen was continued. These dosage regimens of adrenocortical steroids, while sufficient to re-

TABLE I. Responses of Hypophysectomized (Hyphex) Dog K-44 to Test Dose of Insulin (.025 u/kg i.v.): a) in the Untreated State, b) During a Compound F Regimen Alone, and c) During a Combined Compound F-Growth Hormone (GH) Regimen Compared with That of Normal Dogs.

		Hyphex dog K-44		
Time, min.	Normal* composite	Untreated state	Cpd. F† alone	Cpd. F and GH‡
	Blood sugar in mg %			
0	78	66	64	72
	Insulin .025 u/kg intrav. at 0 time			
10	67	55	60	64
20	66	41	54	58
30	74	40	58	61
40	75	41	62	68
50	77	39	71	72
60		39	74	72
75		40	73	72
90		41	73	73
120		45	71	72
150		51	75	71
180		63	77	72
210		63	77	72
240		63	75	73

* Based on 37 insulin sensitivity tests on 18 normal dogs.

† Cpd. F, .83 mg/kg/day, given for 12 days.

‡ " " " " " " 28 " ; GH, 1 mg/kg/day, for 11 days.

† The renal function studies were done in collaboration with Drs. D. P. Earle and S. J. Farber and the findings will be reported elsewhere.

‡ We should like to express our appreciation to: Dr. A. C. Bratton, Jr., of Parke, Davis Co., for Thrombin Topical and "Penicillin S-R"; Dr. R. K. Richards of Abbott Laboratories for Pentothal Sodium and Penicillin G Procaine Aqueous; Dr. K. K. Chen of Eli Lilly and Co. for the Insulin and Duracillin; Dr. E. Alpert of Merck and Co. for "Hydrocortone Acetate" (compound F); and Drs. E. E. Hays and I. M. Bunding of Armour Laboratories for the growth hormone.

store the deranged carbohydrate metabolism of adrenalectomized dogs to normal, nevertheless concomitantly elevated their renal functions far above normal levels(10). In view of this effect, the steroid regimens were considered to be above the physiological range. The growth hormone (lot No. J21609R supplied by Armour Laboratories‡) was given in dosages of 1.0 to 1.5 mg/kg intramuscularly once daily, usually after the afternoon feeding, and continued for 12 to 31 days in conjunction with the adrenocortical steroid regimens. Thus the adrenal steroid regimens were administered for periods totalling 39 to 69 days.

Results. In these studies, all the hypophysectomized dogs were first placed on either cortisone or compound F regimens. The effects produced by these 2 hormones on the dogs' carbohydrate metabolism were similar. Table I records the various responses to the test dose of insulin, 0.025 unit/kg intravenously, of dog K-44, a typical hypophysectomized dog in this series. A compound F regimen, 0.83 mg/kg/day, abolished the insulin hypersensitivity of this dog, *i.e.*, it prevented the marked blood sugar fall seen in the untreated hypophysectomized state and accelerated the recovery of the blood sugar to the postabsorptive levels at a rate similar to that occurring in normal dogs. However, the postabsorptive blood sugar was not elevated in this case at the time that the experiment was performed, although it was frequently observed to be at normal levels during the compound F regimen. The glucose tolerance on the compound F regimen showed the following: 1) abolition of the secondary hypoglycemia which is a characteristic of the untreated hypophysectomized state, 2) no significant rise in the peak of the blood sugar at the termination of the glucose infusion and 3) no shift to the right in the decline of the blood sugar (Fig. 1); thus, a normal glucose tolerance was produced. The adrenaline response of steroid-treated dog K-44 also revealed a parallel improvement and approached the average blood sugar rise seen in normal dogs. Hence, the steroid regimen apparently restored the carbohydrate metabolism of these hypophysectomized dogs to normal (as judged by the above criteria). This was in agreement

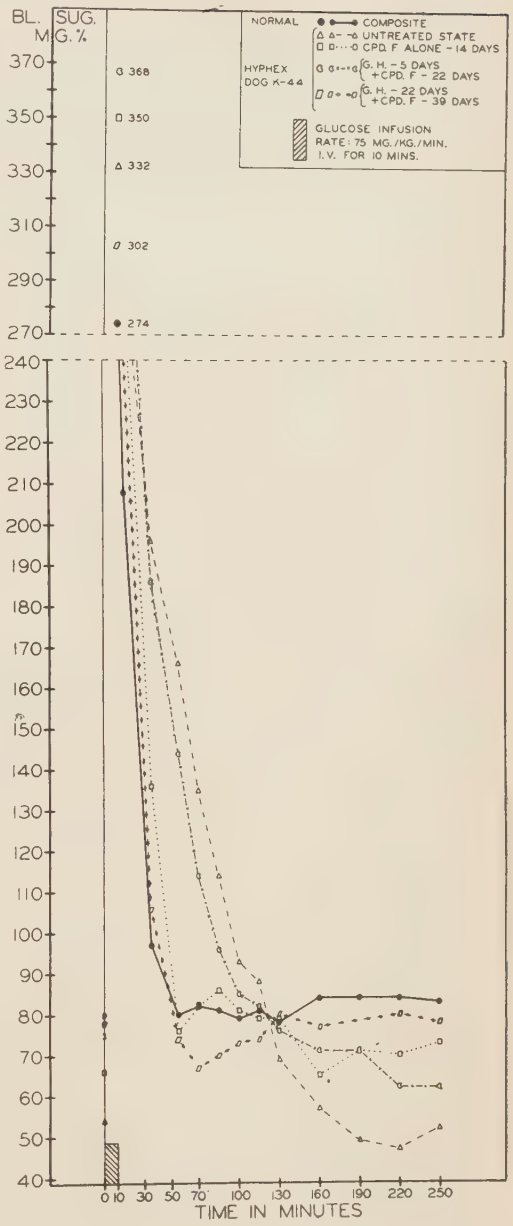

FIG. 1. Blood sugar curves produced by intrav. glucose (.75 g/kg) in a) normal dogs, and in hypophysectomized (hyphex) dog K-44, b) during the untreated state, c) during a compound F (.83 mg/kg/day) regimen alone, and d) during a combined compound F-growth hormone (1 to 1.5 mg/kg/day) regimen. Compound F given for a total of 39 days. Growth hormone Armour #J21609R, 1 mg/kg/day, begun on the 18th day of the compound F regimen and continued for 15 days. Dosage of growth hormone then raised to 1.5 mg/kg/day and given for 7 more days.

with previously published work on the effects of cortisone on the disturbed carbohydrate metabolism of hypophysectomized dogs(11).

At this stage in the course of the adrenocortical steroid regimens, *viz.*, when they had produced a normal carbohydrate metabolism in the hypophysectomized dogs, growth hormone regimens were begun in combination with the steroids and continued for as long as 31 days without the appearance of any toxic manifestations. In the case of dog K-44 a growth hormone regimen, 1.0 mg/kg/day intramuscularly, was begun after the animal had been on a compound F regimen for 17 days. After 11 days on such a combined hormonal regimen (the 28th day of compound F therapy), the insulin response of dog K-44 revealed no change from that seen on the steroid regimen alone (Table I). Likewise, the glucose tolerance of dog K-44 on such a combined hormonal regimen remained unaltered from that obtained on the steroid regimen alone. Fig. 1 records the glucose tolerances of dog K-44 after 5 and 22 days on a combined steroid-growth hormone regimen (the 22nd and 39th days, respectively, of the compound F regimen). Indeed, the glucose tolerance test after 22 days of growth hormone and 39 days of compound F was carried out at a time when the growth hormone dosage had already been increased from 1.0 to 1.5 mg/kg/day for 7 days prior to this test, and yet the glucose tolerance still remained normal. Similarly, the adrenaline response remained at normal levels on the combined hormonal regimen. In some exceptional cases, the maximum blood sugar rise in response to intravenous adrenaline was above the normal range.

Although the responses to insulin and intravenous glucose of these hypophysectomized dogs were restored to normal by the combined hormonal regimens, further tests were performed with larger doses of insulin, 0.25 unit/kg intravenously (10× the test dose) in order to ascertain to what degree, if any, insulin resistance had developed in these dogs during the course of therapy. However, the animals still responded like normal dogs to this large dose of insulin. This result was in marked contrast to the results previously obtained with growth hormone regimens alone in which marked insulin resistance was produced (even to such doses as 1.5 units/kg insulin intravenously)(3).

The Armour growth hormone preparation lot No. J21609R used in the above studies was administered alone to several hypophysectomized dogs and produced complete abolition of their insulin hypersensitivity, *i.e.*, the postabsorptive blood sugar was elevated and practically no fall in the blood sugar occurred after insulin. In addition a marked tendency towards diabetes was noted on all these dogs' glucose tolerance tests and indeed, cessation of administration was necessitated in some dogs in view of the onset of severe toxic signs (anorexia and vomiting) which proceeded eventually in some cases, even to death. Thus, there was no doubt that an active and potent "diabetogenic" growth hormone preparation had been given in conjunction with the various adrenocortical steroid regimens. Reversal of the order of administration of the 2 hormonal agents was planned. In other words, growth hormone was to be given first to hypophysectomized dogs until it would have produced diabetes and insulin resistance. At that time an adrenocortical steroid regimen was to be given in conjunction with the growth hormone regimen. However, this approach was discontinued when after only 7 days administration of Armour No. J21609R, 1.0 mg/kg/day, hypophysectomized dog S-2 died. Dog S-2 was one of the animals that was used in the above studies in which the steroids were given first. Indeed, hypophysectomized dog S-2 had previously received growth hormone Armour No. J21609R, 1.0 mg/kg/day for 31 days, together with a cortisone regimen, 1.0 mg/kg/day, without any ill effects whatsoever.

Discussion. From the above results it can be seen that an adrenocortical steroid regimen (either cortisone or compound F) can restore the carbohydrate metabolism of hypophysectomized dogs to normal, as judged by their responses to insulin, intravenous glucose and adrenaline, without producing insulin resistance, diabetes, or abnormally high adrenaline responses. However, the dosage range employed was deemed in excess of physiological levels in view of the abnormally elevated renal functions of adrenalectomized dogs produced

by these same steroid dosages. Moreover, the administered steroids were augmenting the action of the hypophysectomized dogs' own adrenaline(11) and supplementing their adrenocortical steroid secretions. Nevertheless, when a growth hormone regimen was given in conjunction with the adrenal steroids, no further changes were noted in the hypophysectomized dogs' responses to insulin, intravenous glucose, and adrenaline. Thus, the normal carbohydrate metabolism produced by steroid regimens alone remained unchanged on combined adrenal steroid-growth hormone regimens. The question therefore arose, was the growth hormone preparation used in these experiments contributing at all to the pronounced anti-insulin or, more broadly speaking, blood sugar-raising effect of the adrenal steroids? As mentioned above this growth hormone preparation was an active and potent "diabetogenic" preparation inasmuch as it exerted marked anti-insulin and diabetogenic actions when administered alone to previously untreated hypophysectomized dogs. Furthermore, it was found that the renal functions of these same hypophysectomized dogs which were only partially restored towards normal levels during the steroid regimens alone, were elevated to normal levels during the combined adrenocortical steroid-growth hormone regimens. When the growth hormone regimens were discontinued, the animals thereby receiving only adrenocortical steroids, the renal functions fell from the normal levels to those previously observed during the steroid regimens alone. Thus, this growth hormone preparation exerted a marked effect(12).†

It is evident then, that the combined effect of 2 "diabetogenic" factors (the adrenocortical steroids and growth hormone) was neither a summation nor a potentiation of their individual effects. Furthermore, prolonged administrtion of growth hormone was made possible without the production of diabetes, insulin resistance, and toxic effects, all of which had occurred previously with growth hormone regimens alone(3). It would appear, therefore, that an antagonism existed between these 2 blood sugar-raising agents. The mechanism whereby this antagonism becomes operative is not known. It is known that 1) growth hormone requires a long period after injection into the living animal to exert its action, and 2) purified growth hormone is ineffective when added directly to the isolated diaphragm *in vitro,* but exerts its action in this preparation only if it was injected into the living animal many hours prior to the isolation of the diaphragm. In view of this, it is possible that growth hormone liberates another substance, or else undergoes some transformation. The adrenocortical steroids therefore may inhibit the secretion and/or action of this third substance or block the transformation of growth hormone. Although the results of these experiments using a combined hormonal regimen appear paradoxical at first, it is apparent that in the hypophysectomized dogs these regimens represented a replacement therapy of their absent growth hormone and deficient adrenal steroids. Moreover, the normal organism obviously does not have diabetes and yet has both growth hormone and the adrenal steroids.

The importance of an anterior pituitary factor, most probably growth hormone or some factor closely linked with it,§ in the regulation of carbohydrate metabolism cannot be denied. In previous observations, a comparison of the insulin responses of the adrenalectomized dog and of the hypophysectomized dog revealed a striking difference between the two(13). The adrenalectomized dog, while more sensitive to insulin than the normal dog, was shown to be far less sensitive than the hypophysectomized dog, although the latter animal still had an adrenaline secretion and some adrenocortical steroid secretion both of which were completely absent in the adrenalectomized dog. This pointed to the importance of an anterior pituitary factor as the

§ Raben and Westermeyer have claimed (PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 83) that their growth hormone preparation is nitrogen-retaining with little, if any, diabetogenic activity. These findings as yet have not been corroborated. Whether or not growth hormone or some other anterior pituitary factor is responsible for the actions in carbohydrate metabolism, it is still apparent that an anterior pituitary factor, in addition to ACTH, has a role in the regulation of normal carbohydrate metabolism.

agent responsible for the difference in insulin responses of these 2 experimental animals. The experiments employing a combined hormonal regimen therefore may provide a clue as to the balance prevailing between these 2 blood sugar-raising agents in the hormonal regulation of normal carbohydrate metabolism. It has been suggested that growth hormone, by interfering with peripheral utilization of sugar, raises the blood sugar and this eventually results in the exhaustion of the pancreas(3). To this relationship of growth hormone to the pancreas should be added an important interrelationship of growth hormone with the adrenal cortex on the basis of the above experiments.

Summary. 1. Continued administration of cortisone or hydrocortisone regimens, in dosages (0.83 to 1.2 mg/kg/day) sufficient to restore the carbohydrate metabolism of adrenalectomized dogs to normal, completely abolished the insulin hypersensitivity, secondary hypoglycemia of the glucose tolerance test, and adrenaline resistance of hypophysectomized dogs without the production of insulin resistance, diabetes, or abnormally high adrenaline responses. 2. Continued daily administration of a potent "diabetogenic" growth hormone (1.0 to 1.5 mg/kg/day) in conjunction with the cortisone or hydrocortisone regimens did not alter the carbohydrate metabolism of the hypophysectomized dogs previously observed on the steroid regimens alone, *i.e.*, it remained normal as judged by the above criteria. 3. The insulin resistance, diabetes, and toxic manifestations produced by growth hormone regimens alone were not observed during combined adrenocortical steroid-growth hormone regimens and thus, a more prolonged period of growth hormone administration was made possible. 4. It is suggested that a balance exists between growth hormone and the adrenocortical steroids in the regulation of the carbohydrate metabolism of the normal organism. This balance, in part, may express itself as an antagonism between the two either in the liberation of another factor or in the transformation of growth hormone.

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Attempts to Demonstrate Interference Between Coxsackie and Poliomyelitis Viruses in Mice and Monkeys. (19900)

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Using infant mice Dalldorf(1) demonstrated interference between a group B strain of Coxsackie virus and the Lansing strain of poliomyelitis as evidenced by decreased frequency and prolongation of the course of poliomyelitis, and by survival. On the other hand, Sulkin and Manire(2) reported that the inoculation of Lansing poliomyelitis virus into infant mice offered some slight protection to infection with a group A Coxsackie virus.

No evidence of interference was obtained by Howitt and Nichols(3) when mixtures of Coxsackie and poliomyelitis viruses were given to rhesus monkeys. The present report describes the results obtained with locally and freshly isolated strains of poliomyelitis and Coxsackie viruses occurring in New South Wales during the 1950-51 epidemic of poliomyelitis (Stanley *et al.*)(4) and with the MEF1 strain of poliomyelitis.

Materials and methods. Virus strains. The MEF1 strain of poliomyelitis virus was obtained through the courtesy of the George Williams Hooper Foundation, University of California Medical Center, California. After 10 passages by the intracerebral route in adult mice, the strain was adapted to suckling mice by 7 successive passages by the intracerebral route. The Cobbett strain of poliomyelitis was used in the monkey experiments. This is a Brunhilde-like virus and its properties are described elsewhere (Stanley and Dorman) (5). The Coxsackie viruses used in these experiments comprised both group A (myositis producing) and group B (encephalitis and myositis producing) strains. All strains were isolated in Sydney during the 1950-51 epidemic of poliomyelitis. *Animal stock.* The mice used were bred in this laboratory's animal house and were of the same stock used for earlier investigations. The monkeys were imported from Singapore and consisted of *M. irus, M. nemestrinus,* and *M. mulatta.* All appear to be equally susceptible to infection with Australian strains of poliomyelitis.

Experimental. Experiments with mice. 1) *Effect of cerebral trauma on susceptibility of mice to infection with the MEF1 strain of poliomyelitis by the intravenous route.* Adult mice were inoculated by the intracerebral route with 0.03 ml sterile broth, or 0.03 ml of a 10% suspension of mouse tissue infected with Coxsackie virus. After intervals of 15 minutes, 60 minutes, and 6 hours, 0.01 ml of a suspension of MEF1 infected mouse cords was given by the intravenous route. Eight animals were used in each group, and they were observed for 3 weeks. No paralysis or death was observed, irrespective of whether broth or a Coxsackie virus suspension in broth was used to produce cerebral trauma.

TABLE I. Titration of MEF1 Strain of Poliomyelitis in Adult Mouse Brain in Presence of Group A (S4) and Group B (E46) Coxsackie Virus.

MEF1 virus dilution	MEF1 virus in the presence of	Time in days to paralysis or death: 2	3	4	5	6	7	8	9	10	11	12	13	14	15	No. of mice paralyzed	Avg incubation time, days	Avg incubation time adjusted to a reading of 1 for control group
10^{-1}	Broth	—	3	1	—	—	2	1	—	—	—	—	1			8	6	1
	S4	—	3	2	1	—	—	2								8	4.7	.8
	E46	—	—	—	1	—	—	1	2	2	1	—	1			8	8.7	1.5
10^{-2}	Broth	—	—	1	—	—	—	3	2	2						8	8.3	1
	S4	—	3	1	1	—	1	2								8	5.1	.6
	E46	1	—	—	—	—	—	—	2	1	1	2	—	—	1	8	10	1.2
10^{-3}	Broth	—	—	2	1	1	—	—	2	2						8	7.1	1
	S4	2	—	3	—	1	1	1								8	4.6	.6
	E46	—	—	2	—	—	1	—	1	—	—	—	1	1	1	7	10.5	1.5
10^{-4}	Broth	—	—	—	—	—	—	—	—	1	1	1	2	—	—	6		
	S4	—	—	1	—	—	—	—	2	1	2	1	—	1	—	8		
	E46	—	—	2	—	—	1	—	—	—	1	1	—	—	—	5		
	S4 alone	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0		
	E46 alone	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0		

2) *Titration of the MEF1 strain of poliomyelitis (in the presence of Coxsackie viruses) in adult mouse brain.* A freshly prepared suspension of mouse cord infected with MEF1 virus was diluted 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} 1) in broth, 2) in a suspension of S4 Coxsackie virus in broth, and 3) in a suspension of E46 Coxsackie virus in broth. The S4 Coxsackie virus is a group A strain and the broth suspension used was prepared from infected mouse limbs and used at a dilution of 10^{-2}. The E46 strain is a group B virus and a 10% suspension of infected suckling mouse brain was used as a diluent in this case. Eight adult mice were each inoculated by the intracerebral route with 0.03 ml of each dilution. The mice were observed for 15 days. The results are recorded in Table I. The entire number of 32 mice inoculated with MEF1 + S4 dilutions became paralyzed or died in a shorter interval of time than the mice inoculated with the MEF1 + E46 mixture. In the latter group 28 of the mice developed paralysis. This is suggestive of slight enhancement with the group A strain. Differences between the 3 titrations are more clearly seen by reference to the last column in Table I. In this column the average incubation period has been worked out and adjusted so that the control group always gives a figure of unity. It is seen that with all dilutions of the MEF1 strain the incubation period for group B Coxsackie mice is approximately double that of the group A Coxsackie mice, and the figure for the control is somewhere in between. This would suggest that there is enhancement with group A Coxsackie virus and interference with group B virus.

It was thought that the time interval between the inoculation of the poliomyelitis and the Coxsackie viruses may have some bearing on the demonstration of the interference phenomenon. This was investigated by inoculating mice intracerebrally with Coxsackie virus strain E46, 48 hours before, and 24 hours after, the intracerebral inoculation of the MEF1 strain. No significant alteration in time of onset of paralysis or the number of mice paralyzed could be demonstrated.

One interesting feature of the investigations with the MEF1 strain of poliomyelitis was the

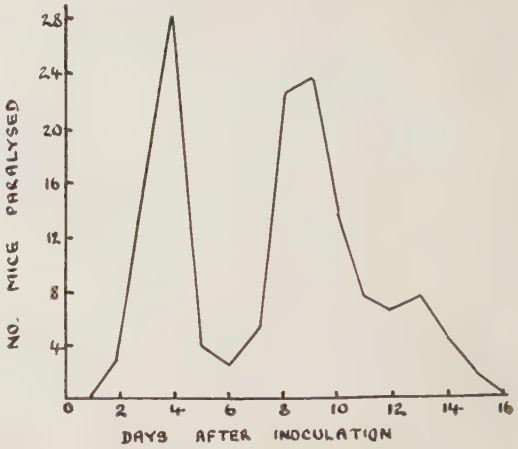

FIG. 1. Mortality of adult mice inoculated by the intracerebral route (.03 ml) with the MEF1 strain of poliomyelitis.

persistent demonstration of 2 peaks in a graph in which the time of onset of paralysis is plotted against the number of animals becoming paralyzed. This is clearly seen in Fig. 1, which was constructed from the data obtained by inoculating 152 adult mice. Although no satisfactory explanation is forthcoming, an attempt to explain the phenomenon was made by inoculating 50 mice intracerebrally with the MEF1 strain. Following this inoculation 5 mice were bled each day. The 5 specimens of sera were pooled each day and inoculated intracerebrally into 5 more mice. Virus could be demonstrated in small amounts in the serum *only* 3 days and 6 days after inoculation. These times immediately precede the 2 periods of highest incidence (Fig. 1).

Experiments with monkeys. Because of the failure to demonstrate very marked changes in the production of poliomyelitis in mice with the MEF1 strain and strains of Coxsackie viruses, similar experiments were planned using monkeys as test animals. One reason for attempting the monkey experiments was that strains of poliomyelitis virus freshly isolated from a human source could be used, whereas the MEF1 strain had been passaged many times in mice, and it was assumed that its virulence for man may have been lessened thereby. The strain of poliomyelitis selected for this work was the Cobbett strain (Stanley and Dorman) (5). Thirty monkeys were used but one monkey died from non-specific causes.

Both poliomyelitis and Coxsackie strains were inoculated in varying concentrations, 1) simultaneously and 2) at different time intervals. All inoculations were made by the intracerebral route. Of the monkeys receiving poliomyelitis virus alone 10 out of 10 became paralyzed with an average incubation period of 8.2 days. Nothing abnormal was detected with the 3 monkeys receiving Coxsackie virus alone. Twelve out of 16 monkeys receiving both Coxsackie and poliomyelitis strains became paralyzed with an average incubation period of 9.6 days. The 4 animals not showing paralysis were challenged 2 months later with 1000 ID_{50} of the Cobbett strain of poliomyelitis. All animals proved resistant to this challenge.

Discussion. It is clear that further investigations must be carried out if any definite interference between the 2 groups of viruses is to be demonstrated. Although Dalldorf(1) showed that Coxsackie viruses of group B exert a "sparing effect" on poliomyelitis infection of mice of different ages, Sulkin and Manire(2) obtained evidence that the inoculation of 3-day-old mice with the Lansing strain protected between 20% and 30% of the animals from a lethal dose of Coxsackie virus (group A). The possibility of an enhancing effect with group A strains is suggested by a close examination of Table I. No such effect could be demonstrated in monkeys. One reason may be that the concentration of group A viruses in the brain of the 2 hosts varies. Without histological evidence of infection, the ID_{50} of suckling mouse brain 3 to 4 days after infection (by the intraperitoneal route) with some group A strains may be approximately 10^{-8}. With monkeys the ID_{50} of brain tissue was 10^{-3} for the same Coxsackie strain. This was determined by removing the monkey brain 6 days after intracerebral inoculation of the virus and making an emulsion of the brain in a known volume of cold broth. This suspension was then cleared and titrated in suckling mice. Evidence that the group B strain of Coxsackie virus prolongs the incubation period of MEF1 poliomyelitis virus in adult mice is in keeping with the findings of Dalldorf(1), who demonstrated a similar phenomenon in younger mice with the Lansing strain of poliomyelitis. The failure to demonstrate interference in monkeys with group B strains confirms the observations of Howitt and Nichols(3), and of Melnick(6), but may be due to the greater resistance of monkeys to infection with Coxsackie viruses.

Summary. The intracerebral inoculation of mixtures of viruses into adult mice indicates that a group A Coxsackie virus decreases the incubation period of the MEF1 strain of poliomyelitis, while a group B Coxsackie virus increases the incubation time. Similar effects could not be demonstrated in monkeys using a strain of poliomyelitis recently isolated from the cord of a fatal human case. Attention was drawn to peculiarities in the time of onset of paralysis with mice infected with the MEF1 strain of poliomyelitis.

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Cultivation of Poliomyelitis Virus in Tissue Culture III. Synthetic Medium in Roller Tube Cultures.* (19901)

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(Introduced by R. C. Parker.)

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We have reported(1-4) that a synthetic nutrient medium, Mixture No. 199, devised by Morgan, Morton, and Parker(5,6) can be used in the cultivation of Lansing poliomyelitis virus. In these studies, the virus was cultivated in Maitland-type (Erlenmeyer flask) cultures of tissue derived from human embryonic brain and spinal cord, human embryonic kidney, tonsils of children, and rhesus monkey testis, kidney, lung, and other organs. It is the purpose of the present paper to report that several strains of the Brunhilde, Leon, and Lansing types can be grown in roller tube cultures of tissue from monkey testis. In these cultures, Mixture 199 has been the only added source of nutriment.

Materials and methods. Preparation, maintenance, and infection of cultures. Methods were based on those introduced by Enders and his associates for the cultivation of vaccinia (7) and poliomyelitis(8) viruses, and later modified by others(9,10). In our experiments, however, Mixture 199 was used as nutrient in place of the more complete feeding mixtures containing such naturally-occurring ingredients as embryo extract, horse serum and serum ultrafiltrate, used by other workers. Testes of immature rhesus monkeys were minced finely in Mixture 199 containing penicillin (100 units/ml) and streptomycin (100 μg/ml). Two drops of reconstituted chicken plasma (Difco) were added to rimless test tubes (15 $\times$ 1.5 cm) and spread evenly over the lower third. Eight to 12 fragments of testicular tissue were then placed on the plasma-lined surface. Clotting was accomplished by the addition of one drop of reconstituted chick embryo extract (Difco). A volume of 2 ml of Mixture 199 was then added to the cultures. Some cultures were also prepared in larger tubes (23 $\times$ 5 cm) to which were added 15 drops of plasma, about 100 fragments of tissue, 5 drops of extract, and 20 ml of Mixture 199. All cultures were rotated mechanically at 37°C. The tissue fragments were examined microscopically each day, and as soon as an abundant outgrowth of fibroblasts was observed, the cultures were infected with virus. The time required for adequate outgrowth of fibroblasts was usually about 7 days; during this period, no fluid changes had to be made because of the buffering action of Mixture 199. The outgrowth of fibroblasts was somewhat less abundant than we have observed in cultures treated with mixtures containing naturally-occurring ingredients. Cultures in the smaller tubes were infected by removing all nutrient and replacing with 2 ml of Mixture 199 to which an appropriate amount of virus-infected fluid had been added; in the larger tubes, the volume was 20 ml. In the beginning, cultures of virus were initiated by adding infected material in the form of mouse CNS (Lansing strain), monkey CNS (Brunhilde, Canadian Eskimo, Y-SK, and Leon strains), or infected tissue culture fluid (Mahoney, MEF1, and Saukett). Subcultivation was continued by inoculating infected culture fluids, removed 16-30 days after initiation of the culture, into 7-day-old cultures of monkey testis. The outgrowths of fibroblasts were examined daily for cytopathogenic changes(8-10). If the object of the experiment was to harvest the maximal volume of virus-infected fluid, changes of nutrient were made every 4 days. If the experiment was one in which virus or serum was being titrated, the fluids were not changed, and the tests were read 6-8 days after the addition of virus or virus-serum mixtures to the cultures. *Titration of viruses and antisera.* Culture fluids infected with some of the strains mentioned were titrated by the inocu-

* Aided by a Grant from The National Foundation for Infantile Paralysis.

lation of 0.2 ml of 10-fold dilutions into 2 or 3 cultures of monkey testicular tissue showing adequate outgrowth of fibroblasts. The titer of a virus suspension was the highest dilution which produced cytopathogenic (degenerative) changes in all of the inoculated cultures within 6-8 days, the fluids not being changed during this period. For convenience, this titer may be designated the CPD (cytopathogenic dose). A refinement of technic would be the inoculation of 8-10 tubes, with the calculation of the CPD_{50}. Poliomyelitis antisera prepared in monkeys in our laboratory were also titrated. Suitable 10-fold dilutions of serum were mixed with sufficient virus-infected tissue culture fluid to give a final amount of virus of 50 CPD. The mixtures were held at room temperature for 1½ hours, then inoculated into 2 or 3 cultures of monkey testis showing adequate outgrowth of fibroblasts. A titration of the virus-infected fluid added to the serum mixtures was performed with each batch of serum tests. The neutralizing endpoint of the antiserum was determined by the highest dilution which completely inhibited the cytopathogenic action of the added virus in 6-8 days, the fluid not being changed during this period. This neutralizing titer may be designated the CPID (cytopathogenic inhibiting dose). Cytopathogenic inhibiting titers are expressed as dilutions of serum before the addition of an equal part of virus, and are not therefore "final" titers. *Immunization of monkeys.* Groups of rhesus monkeys were immunized in order to examine the antigenicity of Lansing and Brunhilde viruses cultivated in the presence of Mixture 199. Four monkeys received every 2 weeks, by the intramuscular route, Lansing virus grown in cultures of monkey testicular tissue in large roller tubes. Six monkeys were likewise injected with tissue-cultivated Brunhilde virus. A third group was inoculated with Brunhilde virus in the form of a pool of infected monkey CNS tissue. The pool used was prepared from the cord enlargements of monkeys harvested on the first day of paralysis following the thalamic inoculation of 0.8 ml of a 1 in 10 suspension of a Brunhilde seed pool. The pool was not titrated in monkeys, but another pool of the same virus prepared by similar methods at about the same time had a PD_{50} titer in monkeys of $10^{-5.2}$ (0.8 ml volumes thalamically). All 3 groups received the virus inoculum incorporated in an equal volume of Freund's adjuvant (without mycobacteria), since it has been found that the use of adjuvant leads to an increase in antigenicity (11-13). The animals were bled at the time of each injection, and the serum was tested for neutralizing antibody in tissue cultures. The details of injections and bleedings, together with antibody levels are shown in Table II.

Results. Propagation in small roller tubes. In cultures of monkey testis prepared with Mixture 199, but not infected with virus, fibroblasts grew out rapidly, and in about 2 to 3 weeks the lower third of the tube was lined by a confluent growth of cells. Cell multiplication continued throughout the 60-70 days that experiments were commonly run. In one series maintained for a longer period, cell division was observed after about 100 days. The pH of uninfected culture fluids was lowered regularly by the metabolizing tissue from 7.8 to about 7.0 in 4 days, for the first 12 to 15 fluid changes. Thereafter, the pH was lowered only to about 7.4. Microscopically, fibroblasts appeared normal for 60-70 days; slight degenerative changes were observed later, but these could not be mistaken for the more severe cytopathogenic changes induced by the virus. In infected cultures, all strains of the 3 types mentioned above induced, in some measure, cytopathogenic changes in the outgrowing fibroblasts. At one extreme, the Brunhilde strain produced extensive cytopathogenic changes, and cell multiplication ceased about 4 weeks after addition of virus. The Mahoney, MEF1, Y-SK, and Saukett strains likewise induced marked cytopathogenic effects. By contrast, in cultures infected with Lansing virus, cell multiplication and degeneration proceeded side by side. Thus, newly divided cells were found in close proximity to cells showing the minor degree of cytopathogenic effect usually associated with the growth of the Lansing virus in tissue culture. The Leon strain likewise induced only minor degrees of degeneration. The Canadian Eskimo strains had characteristics intermediate between those of the Brunhilde

TABLE I. Cultivation of Brunhilde Virus in Monkey Testis with Mixture No. 199, Second Subculture in Large Roller Tubes.*

Fluid changes	Age of cultures (days)	pH of pooled culture fluids on day of change†	Titer of pooled culture fluids (CPD)‡
1	9	7.45	2
2	13	7.45	4
3	17	7.5	3
4	21	7.6	4
5	25	7.6	5
6	29	7.6	5
7	33	7.65	4
8	37	7.65	5
9	41	7.65	3
10	45	7.65	4
11	49	7.75	2
12	52	7.75	3
13	56	7.75	2
14	59	7.8	1
15	63	7.8	1
16	66	7.8	0
17	73	7.8	0

* Inoculum was 50000 CPD of Brunhilde virus (4th fluid change of 1st subculture).

† Fluids were pooled from 2 roller tubes.

‡ Determined in tissue culture as described in text, and expressed as negative logarithms.

and Lansing strains. *Propagation in large roller tubes.* The Brunhilde strain was readily propagated in cultures of monkey testicular tissue treated with Mixture 199. The titers of the culture fluids, and the pH readings of the second subculture of virus are given in Table I. It was found that the pH did not drop as low in cultures in large tubes as in cultures in small tubes. Degenerative changes were observed in the outgrowing fibroblasts, and the appearances were essentially similar to those observed in cultures in small tubes. High titers of virus were produced in culture fluids for 52 days and smaller amounts up to 63 days. Lansing virus was also cultivated in large tubes. The LD_{50} values of the culture fluids, determined by the cerebral inoculation of mice (0.03 ml volumes), were between $10^{-1.0}$ and $10^{-2.0}$, *i.e.*, similar to those reported by others working with cultures in small tubes. *Antigenicity of tissue cultivated virus in monkeys.* Table II presents serum antibody titers in 2 groups of rhesus monkeys immunized with Brunhilde virus, one group having received monkey-passed virus and the other tissue-cultivated virus. It will be seen that 8/12 monkeys developed paralysis attributable to infection contracted from the first of the series of injections given every 2 weeks. None of the monkeys showed Brunhilde antibody before the start of immunization, but high titers were detected in samples removed at 4, 6, 8, 10, and 12 weeks, in both groups of monkeys. In the group immunized with tissue-cultivated virus, antibody titers of 3.0 or 4.0 had developed by the fourth week of the immunization program after only 2 injections of virus. The amounts of virus in these 2 injections were 2,000 and 200,000 CPD, respectively. In the group receiving monkey-passed virus approximately 40,000 PD_{50} of virus were given on each occasion, and these amounts did not appear to result in as brisk an antibody response. Although it is not possible to relate CPD to PD_{50} in absolute terms, it would appear likely that tissue cultivated virus is at least as potent antigenically as an equivalent amount of virus-infected cord tissue. The specific nature of the antibody response was indicated by the complete absence of neutralizing antibody to the Y-SK and Saukett strains in these sera. Satisfactory results were also obtained in monkeys immunized with Lansing tissue-cultivated virus on a similar schedule. Serum titers in individual monkeys were 3.0 or 4.0 at the fourth and sixth weeks. A recent short report by Salk(14) also refers to the employment of tissue cultivated viruses of the 3 antigenic types in the immunization of monkeys.

Conclusions. In experiments in which poliomyelitis virus was grown in cultures of monkey testis in roller tubes, it was shown that the commonly used feeding mixtures containing horse serum and embryo extracts could be replaced by the synthetic nutrient, Mixture No. 199, devised by Morgan, Morton, and Parker(5,6). Such cultures were found to support the growth of several different strains of poliomyelitis virus, and these produced cytopathogenic changes in the outgrowing fibroblasts. It was therefore possible to carry out titrations of viruses and of antisera in roller tube cultures of monkey testicular tissue with Mixture 199. In these titrations, an advantage of the use of Mixture 199 lay in the fact that no fluid changes needed

TABLE II. Serum Antibody Titers of Monkeys Immunized with Brunhilde Virus.

Type of inoculum	Monkey reference No.	No. of inoculations at 2-weekly intervals*	Serum antibody titers (CPID) at following stages of vaccination program† Wk 0	4	6	8	10	12	Outcome of vaccination program‡	Onset of paralysis wk
Monkey CNS	A612	5	0	—	2	2	3	—	NP	2
	613	1	—	—	—	—	—	—	FP	2
	614	1	—	—	—	—	—	—	FP	2
	615	5	0	—	2	2	3	3	NP	2
	616	6	0	2	3	4	4	4	H	
	617	2	—	—	—	—	—	—	FP	1
Tissue cultivated virus	A645	5	0	—	3	4	4	4	NP	1
	646	6	0	3	3	4	4	4	H	
	647	6	0	4	4	4	4	4	H	
	648	1	—	—	—	—	—	—	FP	1
	657	6	0	3	3	4	4	4	H	
	658	5	0	3	3	4	4	4	NP	1

* All injections given by intramusc. route, except first 2 inj. in A657 and A658 given subcut.; all inocula combined with equal parts of adjuvant; wt of cord/inj., .2 g; vol. of tissue culture fluid, 2 ml.

† Titration carried out in tissue culture; titers expressed as negative logarithms.

‡ NP = non-fatal paralysis; FP = fatal paralysis; H = healthy.

to be made during the 6 or 8 days that the titrations were run. The Brunhilde and Lansing strains were grown successfully in large roller tube cultures, and high titers were obtained. The Brunhilde and Lansing strains grown in such cultures stimulated satisfactory antibody levels in monkeys when incorporated with adjuvants and injected by the intramuscular route. The use of Mixture 199 is recommended for studies on the mechanism of the growth of poliomyelitis viruses in tissue culture. The usefulness of the synthetic medium as a nutrient in cultures infected with material derived directly from poliomyelitis patients is currently being investigated by us. Attempts are also being made to grow poliomyelitis viruses in bulk.

Summary. 1. The Brunhilde, Mahoney, Canadian Eskimo, Lansing, Y-SK, MEF1, Leon, and Saukett strains of poliomyelitis virus have been propagated in roller tube cultures of monkey testicular tissue treated with synthetic Mixture No. 199, devised by Morgan, Morton, and Parker. 2. Virus-induced cytopathogenic changes occurred in the fibroblasts and were similar to those observed in cultures treated with mixtures containing naturally occurring ingredients. 3. Virus and serum titrations in roller tube cultures were read 7 days after infection of the cultures; during this period no change of culture fluid was necessary. 4. The Brunhilde and Lansing strains were propagated in large roller tube cultures treated with Mixture 199, and high titers were obtained. 5. Culture fluids infected with Brunhilde and Lansing viruses from large roller tubes (mixed with adjuvants) were used to immunize groups of monkeys by the intramuscular route, and high serum antibody levels (4-0) developed promptly.

We are indebted to Dr. R. C. Parker and his colleagues for supplies of the synthetic medium. We wish to thank Doctors D. Bodian, H. A. Howe, J. L. Melnick, and J. E. Salk for sending us material representing various strains of poliomyelitis virus.

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Stimulation of Peristalsis in the Potassium Deficient Rat by Acetyl-β-Methylcholine.* (19902)

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Potassium deficiency in the rat is accompanied by a decrease in intestinal tone and motility(1-3). This may be due either to a lack of stimulation or to an inability of the smooth muscle of the small intestine to respond to stimuli. Acetyl-β-methylcholine (ABM) has been observed to increase the tone and peristaltic activity of the stomach and small intestine of the dog and of man (4,5), but observations on the *in vivo* effect of ABM on intestinal tone and motility in rats have not been reported.

The experiments reported herein show that ABM increases the propulsive motility of the small intestine of the potassium-deficient rat, but does not affect the peristaltic activity of the intestine of rats receiving a complete diet.

Experimental procedure. The potassium-deficient rats and their controls were fed a purified sucrose-casein diet similar to that of Grunert and Phillips(6). The potassium-deficient diet was analyzed for potassium by means of the Perkin-Elmer model 52A flame-photometer after wet ashing and was found to contain 0.01% potassium. Groups of 10 male and 10 female albino rats of the Sprague-Dawley strain which weighed 100-120 g were placed on the deficient or the control diet. The diets and distilled water were fed *ad libitum*. After 10 weeks on the diet, the intestinal motility of each of the rats was determined by means of a modification(3) of the technic of Macht(7). The *ABM* was given in the form of a freshly prepared distilled water solution containing 1 mg of U.S.P. acetyl-β-methylcholine chloride per ml. The ABM-chloride was stored in a calcium chloride desiccator and weighings were made as rapidly as possible to avoid errors due to its hygroscopicity. A study was conducted to determine the dosage of ABM necessary to produce the effects of parasympathetic stimulation in male rats weighing from 200-250 g and maintained on a "stock-diet."†

Results and discussion. Although ABM stimulated lacrimation and salivation(4,5), it did not cause any increase in the propulsive motility of the intestine as determined by this technic when rats receiving the stock diet were used as the test animals (Table I). It appears that the peristaltic activity of the intestine of the rats fed the stock diet was already adequate and could not be further increased by ABM.

ABM was found to increase the intestinal motility of the potassium deficient rats as shown in Table II. This indicates that the factor limiting peristaltic activity in the potassium deficient rat is a lack of stimuli rather than an inability of the smooth muscle of the

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† Stock diet: 28% ground wheat; 24% ground yellow corn; 10% soy bean oil meal; 12% skim milk powder; 8% alfalfa leaf meal; 5% butter; 2% Wilson's 1:20 liver powder; 1% calcium carbonate; 1% iodized NaCl.

TABLE I. Response of Stock Diet Rats to Various Doses of Acetyl-β-Methylcholine.

mg ABM–Cl/ 100 g body wt	Bolus travel (% of total length)	Effect on lacrimation and salivation
0	65 ± 7*(3)†	0
.02	68 ± 13 (3)	0
.05	67 ± 13 (3)	0
.1	69 ± 19 (3)	L‡
.2	56 ± 16 (3)	L and S
.4	68 ± 14 (3)	Copious L and S

* Stand. dev.
† No. of determinations.
‡ L = lacrimation; S = salivation.

TABLE II. Effect of Acetyl-β-Methylcholine on Intestinal Motility of Potassium Deficient and Control Rats.

Dietary K	Bolus travel (% of total length)	
	Non-treated	.2 mg ABM–Cl /100 g body wt
.01%	26 ± 12*(8)†	66 ± 19 (7)
.5 %	70 ± 18 (9)	72 ± 16 (10)

* and † as in Table I.

intestine to respond. This may indicate neuropathology, a decrease in muscle tone under vagal control, impaired neuromuscular transmission, or a decreased sensitivity of the intestinal musculature to parasympathetic stimulation. *In vitro* studies have shown that potassium stimulates glycolysis which is catalyzed by an extract of rat brain(8), that the addition of KCl to brain slices increases aerobic glycolysis(9), and that increasing the potassium content of Locke's solution results in increased synthesis of acetylcholine by brain slices(10). It has also been observed that the exclusion of KCl from Kreb's solution increases the threshold stimulus for mammalian A nerve fibers(11). These data indicate that potassium has an important role in the physiology of nervous tissue. There is also evidence which indicates that potassium is important in the transfer of nerve impulses across the neuromuscular junction(12) and that potassium sensitizes muscles to acetylcholine(13).

Summary. Acetyl-β-methylcholine was observed to stimulate intestinal motility in potassium deficient rats but did not affect the intestinal motility of rats receiving complete diets.

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Estrogenic Action of Some DDT Analogues.* (19903)

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Burlington and Lindeman(1) found chronic 2,2′-bis-(p-chlorophenyl)-1,1,1-trichloroethane (DDT) administration to effect inhibition of

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† Predoctoral Fellow, National Science Foundation.

TABLE I. Dosage and Estrus Response of DDT and Analogs.

Drug	DDT			DMDT			DHDT				
Dosage in mg	15	30	45	15	30	45	10	15	20	25	45
No. of animals tested	6	6	7	6	6	6	6	6	6	6	6
% animals showing estrus	0	0	0	0	0	0	50	75	100	100	100

testis growth and development of secondary sex characteristics in White Leghorn cockerels. These authors also speculated that the inhibition might be due to an estrogenic action upon the part of DDT since they felt the latter to be structurally similar to diethyl stilbestrol. The present investigation had its origin in the fact that DDT does not fulfill the requirements for estrogenic action as hypothesized previously(2,3) and, therefore, if actually a true estrogen, would represent an especially interesting case for both practical and theoretical reasons. In the light of the working hypothesis relating chemical constitution to estrogenic activity(2,3), DDT does possess a relatively large, rigid, and lipoid soluble molecular constitution, but it does not present active hydrogen atoms approximating the hypothesized optimum distance of 14.5 Å.‡ The present investigation reports, therefore, estrogenic tests carried out upon DDT and some related analogues in the ovariectomized rat. Portions of this report have been presented previously in abstract form(4). Burlington and Lindeman(5), in an abstract presented at the same time, reported new evidence concerning the effects of DDT on the blood of the chicken. Their results confirm the present studies indicating DDT is not estrogenic.

Materials and methods. The substances utilized in these studies, with the exception of DDT (obtained through commerce), were prepared by the conventional chloral condensation with appropriately substituted benzene derivatives(6). The estrogenic tests were carried out as previously described(3) by a method essentially similar to the classical procedure of Allen and Doisey(7).

Results and discussion. The results of the estrogenic tests are detailed in Table I. These data indicate that DDT does not possess an estrogenic action. 2,2′-bis-(p-Hydroxyphenyl)-1,1,1-trichloroethane (DHDT) appears to be an active estrogen while 2,2′-bis-(p-methoxyphenyl)-1,1,1-trichloroethane (DMDT) is inactive at doses twice as large as the minimal 100% estrus dose for DHDT. These results, therefore, indicate that DMDT is not demethylated *in vivo* to any appreciable extent to the active estrogen DHDT.

In the light of the original working hypothesis(2,3) it must be pointed out that the presence of electronegative chlorine atoms in the p,p′ orientations should lead to an inactivation of DDT since these atoms prohibit the existence of active hydrogen at these presumably crucial positions in the molecule. Therefore, under the working hypothesis, unless these chlorine atoms could be metabolized to groups possessing active hydrogen, DDT should be inactive as an estrogen. That DHDT is an estrogenic agent possessed of such active hydrogens, in conformity with the working hypothesis in this series, supports the conjecture that the lack of estrogenic activity on the part of DDT is not due to the impossibility of obtaining estrogenic activity in this general structure class.

While DHDT is probably not the only active estrogen derivable from DDT it is of special theoretical interest in the general problem concerning the relation between chemical constitution and estrogenic activity. In particular, one notes that while DHDT is not especially active in comparison with diethyl stilbestrol or estradiol, the comparison of DHDT to its non-halogenated congener 2,2′-bis-(p-hydroxyphenyl) ethane(8) shows that the presence of three chlorine atoms confers a seven-fold increase in potency on a molar basis. This increase in activity of DHDT over its non-halogenated congener may be attributed to at least 3 possible factors consistent with the original working hypothesis. The first effect is noted upon comparison of

‡ All model measurements herein reported refer to values obtained through the use of Fisher-Hirschfelder-Taylor atomic models.

the Fisher-Hirschfelder-Taylor atomic models of the analogues. The greater effective volume of trichloromethyl over methyl tends to maintain the benzene rings in a more nearly rigid configuration by virtue of steric blocking of rotation of the benzene rings. Secondly, by virtue of an inductive effect through the resonating ring system, the chlorine atoms would tend to increase the activity of the p,p′-hydroxyl groups. This latter effect may not markedly influence activity, however, since the trichloromethyl group is not attached directly to the ring but is 'insulated' by one aliphatic carbon atom. Much more effective in increasing estrogenic potency would be the influence of the trichloromethyl group on the p,p′-hydroxyl hydrogens by inductive action through space via the field effect.

In conclusion it must be pointed out that the distance between the active hydrogens of DHDT can never achieve the optimum (14.5 Å) and one would not anticipate activity approaching that of estradiol or diethyl stilbestrol.

Summary. 1. DDT and 2,2′-bis-(p-methoxyphenyl)-1,1,1-trichloroethane possess no estrogenic activity in ovariectomized rats in total doses up to 45 mg. 2. 2,2′-bis-(p-Hydroxyphenyl)-1,1,1-trichloroethane possesses seven times the estrogenic activity of its non-halogenated congener 2,2′-bis-(p-hydroxyphenyl)-ethane and this increase in potency may be related to field and steric effects exerted by the trichloromethyl group.

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Effect of Factors other than Choline on Liver Fat Deposition.* (19904)

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The effects of certain amino acids on the metabolic relationship between tryptophan and niacin have been studied extensively (1-6). Recently we were surprised to observe fat deposition in the livers of animals receiving similar diets to those used in the above studies. Since the basal ration contained 0.1% choline and since methionine failed to prevent the occurrence of fatty livers, it appeared that other factors in the ration were involved.

In this paper, we wish to present experiments which demonstrate certain effects of proteins, amino acids and carbohydrate on liver fat deposition.

Experimental. Three-week-old male Sprague-Dawley rats kept in individual cages

TABLE I. Basal Ration for Fat Deposition Studies.

Component	Level in diet (per 100 g ration)
Casein	9 g
L-cystine	.2
Sucrose or dextrin	81.9
Corn oil	5
Salts IV(7)	4
Thiamin • HCl	.2 mg
Riboflavin	.3
Pyridoxine • HCl	.25
Ca pantothenate	2
Choline chloride	100
Inositol	10
Biotin	.01
Folic acid	.02

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TABLE II. Effect of Various Supplements to Sucrose Basal Ration on Liver Fat and Growth Rate.

Supplement	No. of animals	50 mg DL-tryptophan	1.5 mg niacin	% fat in rat livers — Wet basis Avg	Wet basis Range	Dry basis Avg	Dry basis Range	Avg growth rate (g/wk) Avg	Avg growth rate (g/wk) Range
0	9			8.8	(4.4-13.3)	20.4	(12.7-28.4)	14.4	(10.4-20.8)
0	9	+		9.3	(6.6-15.9)	23	(13.5-37.8)	19.8	(15.2-26.4)
0	9		+	13.4	(9.5-19.9)	32.3	(24.4-41.7)	16.4	(8.8-20.8)
6% gelatin	6			2.9	(2.2- 3.8)	8.3	(6.2-10)	3.2	(1 - 6.6)
	6	+		4.8	(3 - 9.6)	11.9	(8.4-15.2)	25.6	(19.2-35)
	6		+	3.7	(3 - 4.4)	11.1	(8.9-14)	25.6	(14.2-38.8)
.16% threonine	9			3.2	(2.3- 4.7)	8.7	(6.5-10.1)	4.4	(.8- 7.6)
	9	+		6.6	(3.8-13.5)	17.5	(11.3-26.6)	23	(14.8-29)
	9		+	7.3	(5.1-15.1)	19.7	(15.1-35.5)	20	(12.2-35.2)
.31% threonine	6			2.4	(2.3- 2.5)	6.9	(6.4- 7.4)	3.2	(2.2- 4)
	6	+		6.2	(5.9- 6.9)	16.1	(15 -18.1)	22	(21.8-22.4)
	6		+	6.5	(5.7- 7.9)	17.2	(15 -19.9)	22.8	(19.2-26.2)

were fed *ad libitum* and were weighed at weekly intervals. The basal diet to which supplements were made at the expense of the carbohydrate is shown in Table I. This basal diet contained cystine in all cases except where indicated. The fat-soluble vitamins were administered orally in the form of 2 drops of halibut liver oil each week. The animals were sacrificed at the end of a 5-week period and the livers of the animals were removed and analyzed for fat content using essentially the procedure outlined by the Association of Official Agricultural Chemists(8). The livers were dried at 50°C in a vacuum oven for moisture determination, and the fat content was determined on duplicate samples of the dried livers. Samples were ground with sand and extracted with ether for 16 hours on Goldfisch extractors. After evaporation of the ether the fat samples were heated in a 100°C oven for 15 minutes, cooled in a desiccator, and weighed to constant values.

Results. The data in Table II show the effect of various supplements when added to sucrose rations containing 9% casein with 0.2% L cystine. When either 50 mg % DL tryptophan or 1.5 mg % niacin was added to this basal ration the growth rate increased but no protective effect upon liver fat deposition was noted. When 6% gelatin was added to the basal ration, however, a decided depression of liver fat together with a reduced growth rate occurred. Either tryptophan or niacin, added with the 6% gelatin supplement, stimulated growth over the controls while the liver fat was still significantly lowered. From this experiment, it appeared that the factors controlling growth and liver fat deposition were not the same. When 0.15% DL threonine was added to the basal ration, growth was inhibited as much as that caused by 6% gelatin and the liver fat was lowered to the same level. When the indicated amounts of either tryptophan or niacin were added to the threonine supplement, growth was increased markedly, but no depression of liver fat occurred. The growth depression was undoubtedly due to an amino acid imbalance(4). Either niacin or tryptophan alleviated this growth depression but an increase in liver fat still occurred.

Griffith and Nawrocki(9) have reported intensification of a choline deficiency when threonine was added with cystine to a low casein diet. The influence of threonine was believed to be due to a direct stimulation of growth or metabolism rather than an antagonistic action towards labile methyl. This seems to be the case since 0.1% choline was present in the basal ration under our conditions. The growth depression which we observed is probably due to a tryptophan-niacin deficiency, yet when either of these factors was added to bring about a growth response, fatty livers occurred. When the level of threonine was doubled as in the last experiment in Table II in the presence of either tryptophan or niacin, growth was restored and liver lipids were reduced, but not to the extent brought about by the gelatin supplement

TABLE III. Effect of Various Supplements to Dextrin Basal Ration on Liver Fat and Growth Rate.

Supplements	No. of animals	50 mg DL-tryptophan	1.5 mg niacin	% fat in rat livers — Wet basis Avg	Wet basis Range	Dry basis Avg	Dry basis Range	Avg growth rate (g/wk) Avg	Avg growth rate (g/wk) Range
0	6			4.9	(3.7-6)	13.9	(11.2-17.6)	21.8	(14 -26.8)
0	6	+		3.9	(3.2-5.2)	11.4	(9.4-14.7)	17.8	(9.2-26.2)
0	6		+	5.9	(5.3-6.5)	16.3	(14.2-18.5)	22.4	(16.2-26)
6% gelatin	6			3.1	(2.5-4.1)	9.3	(7.2-12.5)	7.6	(6 - 9)
	6	+		4.2	(3.5-4.7)	12.9	(11.2-14.1)	22.8	(14.8-30.4)
	6		+	3.2	(2.4-4.4)	9.6	(8 -12.2)	22	(12.8-32.6)
.18% threonine	3			3.9	(3.3-5)	8.9	(7.7-10.7)	11	(5.4-15.6)
+.24% phenyl-	3	+		3.1	(2.4-4.1)	8.9	(7.2-11.3)	31.4	(10.4-32.4)
alanine	3		+	4.5	(4.1-5.3)	13.5	(12.6-15.2)	23.6	(18.8-26)
2% acid-hydro-	3			4.3	(2.9-6.4)	12.8	(8.8-18.8)	15	(13.6-17.6)
lized casein+	3	+		5.1	(4 -5.8)	15.1	(12.2-17.6)	30	(27.6-33.8)
1.5% glycine	3		+	5.6	(5.4-5.7)	16.8	(16.5-17.1)	23.4	(19.2-27.8)

alone. It is clear that gelatin exerts an effect beyond the action of threonine.

In Table III similar data are given when dextrin rather than sucrose was the carbohydrate. The controls on the dextrin basal showed a higher growth rate even without additions of niacin or tryptophan. At the same time it can be noted that the deposition of liver fat was not as severe as in the sucrose group. The addition of either tryptophan or niacin in the indicated quantities failed to promote increased growth over the controls. When 6% gelatin was added to the basal diet, a drop in liver lipids and growth rate was observed. Supplements of either tryptophan or niacin together with gelatin completely overcame the apparent growth antagonism precipitated by the gelatin and maintained the liver fat at low levels. When 0.18% DL threonine and 0.24% DL phenylalanine were supplemented to the basal ration, a similar effect was noted. The addition of either tryptophan or niacin restored the growth without affecting the liver fat. In the final experiment in Table III, 2% acid-hydrolyzed casein and 1.5% glycine were added together to the basal ration. The growth was depressed but liver fat was only slightly altered if at all with respect to the controls. Tryptophan or niacin increased growth and liver fat deposition slightly. The dextrin basal rations supported growth rates of control animals which were much greater than the sucrose controls as has been previously observed in this laboratory by Teply and coworkers(10). On the other hand, it can be observed that the lipid contents of the livers of the dextrin controls were lower. Gelatin exerted a greater effect when sucrose was the carbohydrate.

McHenry(11) cited the roles of biotin, thiamin and carbohydrate in the production of fatty livers. More recently Harper and Katayama(12) have studied the utilization of sucrose or corn starch for growth when added to low casein diets. They showed that corn starch supported better growth than sucrose in a 9% casein basal ration supplemented with methionine. This was also true when methionine was not added to the basal diet. One can surmise that the differences between the 2 carbohydrates may be in the rate of passage of the diet through the gut. A greater availability of the amino acids in the casein might be suspected if the ration passed through the intestine more slowly. The observations by Mulford and Griffith(13), and Salmon(14) that cystine supplementation aggravates renal damage and fatty infiltration of the livers of rats receiving 8% casein diets deficient in choline; and Griffith and Nawrocki(9) that further intensification of the choline deficiency occurred when threonine was added with cystine, prompted us to carry out a third set of experiments which are reported in Table IV. We decided to omit cystine from the basal ration and test the effects of certain protein and amino acid supplements. The most striking effect of the cystine omission may be seen in the control group where the growth was about 50% lower

TABLE IV. Effect of Increasing Protein and Single Amino Acids Supplemented to Sucrose Basal Ration Without Cystine.

Supplement, %	No. of animals	% fat of dry liver		Avg growth rate (g/wk)	
		Avg	Range	Avg	Range
0	6	29	(13.9-35.2)	7.6	(5.8-11.6)
3 Casein	6	11.5	(6.9-17)	15.6	(12.6-18.8)
6	6	10.2	(7 -15.2)	22.6	(16.2-24.8)
3 Gelatin	6	17.2	(9.7-25.8)	10.4	(8.8-12.2)
6	6	13.9	(9 -18.4)	10	(6.2-13.8)
.3 L Proline	6	23.6	(14.8-35.8)	6.6	(1.8- 9.2)
.6	6	24.5	(17 -25.4)	7.2	(4.8-10.4)
.6 DL Alanine	6	23.1	(15.6-28.1)	5.6	(4.2- 8.4)
.3 DL Methionine	3	26.7	(23.6-29.6)	14.9	(10 -17.6)

than similar controls in Table II where cystine was included. It is likely that the sulfur amino acids were limiting for growth in the 9% casein rations(4). Larger amounts of casein added to the basal diet increased growth and decreased liver fat. This, however, was not true in the case of gelatin. Increasing levels of gelatin failed to stimulate growth proportionately to the level added to the diet, but did decrease liver fat proportionately. L proline was chosen as an amino acid supplement because of its high concentration in gelatin and DL alanine was chosen because it showed a slight effect upon the reduction of liver lipids in preliminary trials. The supplements of 0.3% L proline and 0.6% DL alanine are comparable and each decreases the liver fat slightly while the growth in either group is similar to the controls. This observation strengthens the earlier conclusion that those factors controlling the liver fat deposition are not necessarily the same as those controlling growth. To show this more clearly, 0.3% DL methionine was added to the basal ration which resulted in a restoration in the growth equivalent to that exerted by cystine in the control group presented in Table II. While the addition of methionine to the basal ration without cystine increased growth 100% over the controls, it exerted no effect upon the liver fat deposition. It appears that 0.3% DL methionine improved growth without antagonizing liver fat deposition as is often the case when added cystine is present(9,13-15).

The fatty liver which we have obtained under our conditions seems to be one which may be influenced by at least 3 factors: protein, amino acids and carbohydrate. There has been much conjecture as McHenry(11) has pointed out, upon the role of the pancreas in preventing fatty livers. Some observations suggest the essential factor in the pancreas to be the hormone, lipocaic, while other workers claim to have ruled out the hormone action and assert the idea that the proteolytic enzymes from pancreatic juice are necessary for the complete digestion of dietary protein which then reduces fat deposition by some mechanism; perhaps one which involves one or more amino acids specifically. It seems likely from our data that, under our conditions, carbohydrate may effect liver fat and growth by controlling the rate of absorption of the necessary amino acids from the gut by affecting the rate of passage of the diet through the gut or affecting the microorganisms for production of necessary vitamins, or both. Whole protein, such as casein or gelatin, apparently supplies necessary amino acids for controlling liver fat deposition. While certain individual amino acids exert a slight effect on the reduction of liver lipids, it appears that combinations of these amino acids are necessary to bring about a greater reduction in liver fat deposition. (Harper, Monson, Elvehjem work in progress.)

Summary. A number of factors under our experimental conditions influence fat deposition in the liver: the availability of niacin to the animal; the type of carbohydrate used in the ration and type and level of protein used. Individual amino acids and mixtures of amino acids which support optimal growth conditions do not produce minimum levels of fat

in the liver.

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Fecal Electrolytes and Nitrogen During Cortisone or ACTH Therapy.*† (19905)

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In view of the established effects of cortisone, ACTH, and DOCA on the excretion of certain body constituents through the kidney and the skin(1-6), the possible influence of two of these agents on the composition of stools has been studied in a series of patients. Compound E, 50 to 1000 mg per day, or ACTH, 10 to 100 mg per day administered for 3 to 50 or more days, did not produce any characteristic alteration in the output of electrolytes and nitrogen. Collection of control data to establish this fact has incidentally provided information about the amounts of sodium, potassium, chloride and nitrogen excreted in feces during maintenance of a miscellaneous group of subjects on certain types of milk diets.

Materials and methods. Stools have been collected from hospitalized children and adults while on one of the following dietary regimens supplemented by vitamins and iron: a) essentially complete sodium restriction, *i.e.,* low-sodium milk fortified with carbohydrate and milk protein, providing an adequate nitrogen and caloric intake, or b) a whole milk diet similarly modified. Patients with gastrointestinal disorders were excluded, but the available clinical material made it impossible to limit the study to any one category of patients with respect to age or disease. This study is therefore based upon analyses of stools from a miscelleneous group of children and adults ill with rheumatic fever, diabetes mellitus, rheumatoid arthritis, leukemia, muscular disorders, *et cetera.* The daily intake of formula usually exceeded two liters but the seriously ill patients at times took less than the prescribed amount.

The *output* of sodium, potassium, chloride and of nitrogen in formed stools has been measured in control studies as well as during periods of therapy with cortisone or ACTH. Water was taken as desired. The beginning and end of a stool collection period, usually

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3 to 7 days in length, were marked by administering carmine red, 0.3-0.6 g, in a gelatin capsule. Stools colored red by the first capsule were either discarded, or if a part of a series, assigned to the preceding interval, whereas those marked by the second dose of the dye were added to the current collection. Particular care was taken to exclude any unformed or diarrheal stools as well as those contaminated with urine. During the collection period and thereafter the specimens were refrigerated in tared and stoppered bottles. Following determination of the stool weight approximately 200 ml of concentrated sulfuric acid was added to each specimen. They were stirred and permitted to digest overnight. Distilled water was then added in amounts sufficient to produce a homogenous thin suspension with the aid of either a Waring blendor or an Osterizer and the final volume measured to establish the dilution factor. Immediately thereafter duplicate or multiple aliquots of the suspended stool up to 6 in number were pipetted into appropriate vessels for processing and analyses. The procedure developed by Wallace was used in preparing the specimens for sodium and potassium determinations(7,8). Approximately 3 ml of concentrated nitric acid were added to the beaker containing the aliquot of suspended stool together with sufficient distilled water to make a final volume of less than 100 ml. The mixture was then boiled for ten minutes, cooled, diluted to 100 ml and filtered. Suitable dilutions to which lithium sulfate had been added were then analyzed on a Barkley internal standard flame photometer with an experimental error of 2% or less(8). The Hald modification of the silver nitrate method was employed for the stool chloride analyses (9). The procedure was simplified in the later studies by allowing the digestion to occur in tubes placed in boiling water for 30 minutes. In those instances in which an excess of potassium permanganate had been introduced superoxol was added and the boiling repeated. Comparison of this modification with the usual digestion of individual tubes over an open flame indicated that the accuracy of the method was unaffected. The stool nitrogen was measured by means of the macro-Kjeldahl technic in which duplicate analyses usually agree within 2%(10). *Stool electrolyte excretion* has been expressed in milliequivalents per day, in milliequivalents per 100 g of wet stool, and in milliequivalents per g of stool nitrogen. Nitrogen excretion has in turn been presented in grams per day and per 100 g of stool mass. Inspection of the results of analyses of stools collected prior to or following hormone therapy with those obtained during therapy in the same individuals revealed no trends in any of the constituents under study. The results have therefore been pooled in accordance with the dietary and treatment regimens which have been outlined and submitted to conventional statistical treatment. After exclusion of the one or two values more than 3 S.D. away from the mean present in most of the categories, the mean and S.D. were obtained in the usual manner. A "p" value of less than 0.05 was taken to represent a statistically significant difference.

Results. From Tables I and II it is readily evident on comparison with control values that cortisone and ACTH, alone or in combination, exerted little or no influence on the composition of the feces. The statistically significant changes recorded in stool nitrogen and chloride in the low-sodium milk group receiving cortisone and in stool chloride in those treated with ACTH (Table I) are of negligible magnitude.

Inspection of the control data in Tables I and II reveals that during either regimen the fecal potassium is approximately 5-fold greater than the sodium, and that the excretions of these 2 ions are of the same order of magnitude irrespective of the milk formulae used. The stool chloride is somewhat greater on whole milk in which considerably more chloride is present. The rate of loss of nitrogen in feces is the same in each group, averaging 0.8 g per day and slightly more per 100 g of stool.

Discussion. Cortisone and ACTH did not alter the output of electrolytes or of nitrogen in this group of miscellaneous patients. This means of course that the gastrointestinal tract, at least in terms of its ultimate output, does not respond, as do the kidneys or the sweat glands, by a diminished sodium output and a

TABLE I. Stool Electrolytes and Nitrogen on a "Sodium-Free Milk Formula"† Without and With ACTH or Cortisone Therapy.

Therapy		Na			K			Cl			N	
		meq./d.	meq./100 g	meq./1 g N	meq./d.	meq./100 g	meq./1 g N	meq./d.	meq./100 g	meq./1 g N	g/d.	g/100 g
0	Mean	1.3	2.3	2.3	8.1	12.3	12.2	.7	.8	.9	.8	.9
	No.	44	48	46	47	48	46	46	45	44	48	46
	S.D.	1.1	2.1	2.1	5.3	8.2	7.7	.7	.5	.6	.4	.3
Cortisone	Mean	1.3	1.6	2	10.7	12.8	11.5	.5	.6	.5*	1	1.2*
	No.	27	26	25	25	26	22	24	24	21	25	24
	S.D.	.6	.7	1.5	7	5.1	4.5	.5	.5	.5	.8	.4
ACTH	Mean	1.5	1.6	1.6	8.7	8.5	10.9	1.4*	1.4*	1.8*	.8	1
	No.	16	15	11	16	14	11	13	11	9	11	11
	S.D.	1.1	1.1	1.1	5.7	5	7.1	1.4	1.1	1.4	.5	.3
Cortisone + ACTH	Mean	.8	1.8	1.2	7.8	18.2	16.6	.6	.6	.4	.7	1.2*
	No.	6	6	6	6	6	6	3	3	3	6	6
	S.D.	.8	1.3	1.2	4.6	12.4	13				.6	.4

* "p" values less than .05.

† Composition of low-sodium milk formula: Na = 1.1 meq./l; K = 44.9 meq./l; Cl = 18.5 meq./l; N = 5.3 g/l.

TABLE II. Stool Electrolytes and Nitrogen on a Whole Milk Formula* Without and With Cortisone Therapy.

Therapy		Na			K			Cl			N	
		meq./d.	meq./100 g	meq./1 g N	meq./d.	meq./100 g	meq./1 g N	meq./d.	meq./100 g	meq./1 g N	g/d.	g/100 g
0	Mean	1.9	2.3	2.4	12.2	14.6	14.6	1.3	1.2	1.4	.8	1
	No.	38	38	35	40	38	34	38	37	36	38	38
	S.D.	1.4	1.6	1.4	6.8	7	5	1.3	.7	1	.5	.4
Cortisone	Mean	1.7	2.3	2.9	10	13.9	18	.7	.9	1.1	.7	.9
	No.	15	14	15	15	14	15	14	13	12	15	13
	S.D.	.8	1.1	1.7	5.7	6	8.8	.5	.6	.8	.6	.2

* Composition of whole milk formula (based on 30 analyses): Na = 26 meq./l; K = 37 meq./l; Cl = 31 meq./l; N = 5.4 g/l.

greater loss of potassium. It should be pointed out however that our data do not obviate the possibility of very marked effects of adrenocorticotropic hormone or cortisone on electrolytes and nitrogen in the upper portions of this tract with subsequent cancellation of these changes in the lower bowel. As a matter of fact indirect evidence has been presented indicating that in clinical situations characterized by undue sodium retention, exchange resins may not exert their maximal effect(11). It has been postulated that this reflects an increase in adrenocortical activity or effects.

Summary and conclusions. 1. Cortisone and adrenocorticotropic hormone did not produce any characteristic change in fecal electrolytes and nitrogen in a group of miscellaneous patients maintained on milk formulae of restricted or unrestricted sodium content. 2. Data have been presented on the fecal excretion of sodium, potassium, chloride, and nitrogen during maintenance of such patients on a whole milk or sodium-free milk formula.

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Factors Related to Growth of Psittacosis Virus (Strain 6BC) III. Uracil and Related Compounds.* (19906)

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Previous observations(1-3) indicate that pteroylglutamic acid and/or citrovorum factor are required for the intracellular proliferation of psittacosis virus and that certain purines (adenine, guanine) and other compounds are important for the intracellular multiplication of psittacosis virus (6BC). Since these various substances are concerned with the intracellular synthesis of nucleic acids, of which desoxyribonucleic acid (DNA) has been shown to be a prominent constituent of the psittacosis group of viruses(4), it was of interest to see if evidence could be obtained that the pyrimidine uracil, a material substituent of ribonucleic acid (RNA), is related to the intracellular proliferation of psittacosis virus.

Methods. Tissue cultures were prepared by planting whole minced tissues of 9- to 11-day-old chick embryos on perforated cellophane discs(5) in the bottoms of 10 ml Erlenmeyer flasks(2). Two ml of nutrient solution composed of 2 volumes of Hanks's(6) balanced solution and one volume of ox serum ultrafiltrate, to which virus was added, were then placed into each flask. The psittacosis virus (6BC) had been cultivated in the yolk sacs of embryonated eggs for a long period of time. Tissue cultures were incubated at 36°C. After 24 hours the infected nutrient fluid was withdrawn and replaced with 2 ml of fresh nutrient solution to which one of the various compounds had been added. The compound was added to the fluids for 2 successive changes after which normal nutrient solution was employed. Fluid changes were carried out every 4 days and the fluid removed was promptly tested for its virus content by injection into embryonated eggs. A single dilution of the virus-containing fluid from each tissue culture was prepared in broth and 0.25 ml of the material was injected into the yolk sacs of 12 or more 7-day-old embryonated eggs. Eggs were candled daily, deaths recorded, and the average day of death calculated. The virus titer was determined from this value by the method described(7).

To evaluate the possible toxic action of the test compounds on chick embryo tissues, 8

* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service, and by a contract between the Office of Naval Research, Department of the Navy, and the University of Rochester.

fragments of heart tissue from 12- to 14-day-old embryos were planted in the plasma clot lining the walls of roller tubes which were then incubated for 4 days at 36°C while revolving in a roller-tube apparatus. Daily examinations were made to note the capacity of the fragments to pulsate and the outgrowth of fibroblasts at the periphery of the explants.

Results. Of the compounds tested, thiouracil† produced significant suppression of virus growth as shown in the growth curve (Fig. 1). This inhibition was demonstrable at a concentration (0.5 mg/ml) which produced no obvious toxic effects on the chick embryonic heart tissue as measured by contractility and fibroblastic proliferation. 6-Methylthiouracil at the tolerated concentration of 0.1 mg/ml produced slight inhibition of virus growth (Table I). The other compounds, 6-methyluracil, diazouracil, and uracil had no significant influence on virus growth at the concentrations used.

Discussion. Previous studies(1-3) have suggested that the synthesis of DNA is an important event in the multiplication of psittacosis virus (6BC) as would be anticipated, since these viruses contain significant amounts of this substance. These earlier experiments gave direct evidence that interference with intracellular metabolism of the purines adenine and guanine would result in the suppression of virus growth. The data presented here indicate that thiouracil blocks some aspect of uracil metabolism which is normally essential for the formation of virus. Experiments with tobacco mosaic virus(8) indicate that this may be due to interference with the incorporation of uracil into the virus particle. The demonstration that interference with the intracellular metabolism of its substituent uracil suppresses virus growth suggests that the synthesis of RNA is involved in the growth of psittacosis virus unless the uracil has some other role unrelated to RNA.

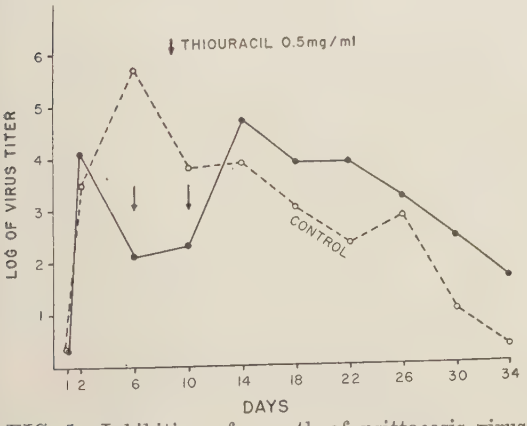

FIG. 1. Inhibition of growth of psittacosis virus (6BC) in tissue culture by thiouracil.

† Provided through the courtesy of Dr. J. M. Ruegsegger, Lederle Laboratories Division, American Cyanamid Co.

TABLE I. Effect of Various Uracil Derivatives on Growth of Psittacosis Virus (6BC) in Tissue Culture.

Compound	Conc., mg/ml	Virus inhibition	Tissue toxicity
Thiouracil	.5	+++	0
6-Methylthiouracil	.5	NT	+++
	.1	+	0
6-Methyluracil	1	+	±
	.5	±	0
Diazouracil	.5	0	0
Uracil	.5	0	0

NT = Not tested.

It is important to note in the experiments here reported that the suppression of virus growth in the tissue cultures was exhibited under conditions which gave no evidence of serious toxic effects on the host tissue cells since fibroblastic growth occurred and fragments of heart tissue continued to pulsate, indicating that their energy metabolism was not seriously impaired.

Summary. Thiouracil inhibited the growth of psittacosis virus (6BC) in tissue cultures at concentrations which had no obvious toxic effects on the host tissues. In tolerated amounts 6-methylthiouracil, 6-methyluracil, diazouracil, and uracil had no effect on virus growth. These experiments suggest that some aspect of the intracellular metabolism of uracil is essential for the multiplication of psittacosis virus (6BC).

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Metabolic Demethylation of 3,5,5-Trimethyl-2,4-Oxazolidinedione (Trimethadione, Tridione).* (19907)

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Methyl groups are now known to be removed metabolically from nitrogen in a wide variety of chemical types of N-methyl compounds. As some of these compounds find extensive use as drugs and as the metabolic products are in some cases themselves pharmacologically active, the reaction of demethylation has important practical implications in therapeutics. From analogy with the closely related derivatives of barbituric acid and hydantoin, it could be expected that the antiepileptic drug, trimethadione, would undergo demethylation in the mammalian body. The product of the reaction would be 5,5-dimethyl-2,4-oxazolidinedione (henceforth to be designated "DMO"). The present report concerns the isolation of this substance from the urine of dogs receiving trimethadione.

Materials. Samples of DMO were supplied through the kindness of Dr. Roger W. Stoughton of Mallinckrodt Chemical Works and Dr. M. A. Spielman of Abbott Laboratories. The compound melts at 76-78°C. The ionic form absorbs strongly in the ultraviolet, the absorption peak lying at wavelengths below 210 mμ. The molar extinction coefficient is 1.1 x 10^4 at 215 mμ and diminishes sharply with increasing wavelength. In this spectral region the absorption of the undissociated molecule in solution in negligible. At 37°C and at an ionic strength of 0.1 the pK′ of DMO as calculated from spectrophotometric data is 6.2.†

Trimethadione was isolated from commercial capsules and recrystallized from water and from petroleum ether. It melted at 44-46°C. At wavelengths longer than 215 mμ the absorption of trimethadione in aqueous solution is negligible in comparison with that of the ionic form of DMO. A test was made for the possibility of contamination with significant amounts of DMO by examination of the ultraviolet absorption of a buffer of pH 8 shaken with an ether solution of trimethadione. In this way it was demonstrated that the sample of trimethadione could not contain as much as 0.2 per cent DMO.

Administration of drugs. Both dogs of Table I were females. The drugs were given in aqueous solution intraperitoneally, the total dose being divided into two portions, which were administered several hours apart. The solution of trimethadione contained 5 g and that of DMO 10 g per 100 ml. With each dog the trimethadione experiment preceded the DMO experiment by at least 3 weeks.

Isolation of DMO from urine. Urine was collected from the dogs for seven days after the drug injection. The urine was acidified to pH about 2 and extracted with ethyl ether. A batch of urine would be divided into four portions, which would be successively shaken with two portions of ether, each five times the volume of each portion of urine. The ether extract was distilled to a volume of 100 ml and was then extracted with four 25 ml portions of a saturated aqueous solution of $NaHCO_3$. The bicarbonate extract was acidified with hydrochloric acid to pH about 2 and shaken with two 50 ml portions of benzene, which were discarded. The aqueous phase was then

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† The pK′ as determined by potentiometric titration has been reported by Erlenmeyer *et al.*(1) as 6.1.

TABLE I. Recovery of 5,5-dimethyl-2,4-oxazolidinedione (DMO) from the Urine of 2 Dogs Receiving Trimethadione and DMO.

		Dose		Material recovered from urine			
Wt, kg	Drug administered	mM/kg	Total wt, g	Wt, g	m.p., °C	Mixed with authentic DMO, m.p., °C	Yield, %
10.9	T*	7	10.9	1.20	75–78	76–78	12.2†
	DMO	7	9.8	1.27	74–78	74–78	13
11.9	T	7	11.9	.27	74–77	74–78	2.5
	DMO	7	10.7	.90	75–78	75–78	8.4

* T = Trimethadione.

† In another exp. this animal was given the same dose of trimethadione; the recovered yield of demethylated product was only 5.4%.

extracted with four successive 75 ml portions of ether. The ether extract was evaporated in a dish at room temperature. The residue was dissolved in water, and by means of systematic "countercurrent" distribution between water and ethylene chloride it was possible to eliminate considerable amounts of colored materials having partition coefficients higher and lower than that of DMO. The distribution was carried out in such a way that nearly all of the DMO accumulated in the final aqueous fractions. Those aqueous fractions containing the greater part of the desired material were treated with sufficient Norit to remove any remaining color and were then extracted with ether. After evaporation of the ether, the residue was crystallized from benzene. Some fractions were freed of tenacious impurities by sublimation at reduced pressure. No search was made for unchanged trimethadione or for other possible metabolic products.

Results. As is shown in Table I, the demethylated product was isolated from the urine of dogs that had received trimethadione and was identified by melting point and mixed melting point. Further evidence of identity was furnished by a study of the ultraviolet absorption spectrum of the material from the first dog after trimethadione administration. Within the limits of experimental error the spectrum of the urinary product coincided with that of authentic DMO at different values of pH. The pK′ of the urinary product as calculated from spectrophotometric data did not differ significantly from the value found for synthetic DMO.

On later occasions the same dogs were given the same molar dose of DMO itself. The yields recovered from the urine are shown in Table I.

Discussion. Isolation of relatively pure DMO from urine by the present procedure undoubtedly entails large losses. The yields shown in Table I are accordingly of very limited quantitative significance. Nevertheless, comparison of the isolated yields when the N-methyl compound and the non-methylated compound are administered would indicate that the extent of demethylation of trimethadione is considerable. The present experiments do not suffice to permit an evaluation of the role played by DMO in the effects seen after administration of trimethadione. There is reason, however, to believe that DMO is retained in the body for long periods of time. The neurological effects of the doses of DMO given to the dogs in these experiments were evident for many hours and the drug continued to appear in the urine for several days. The very transient effect reported(2) for intravenous doses of DMO in mice is probably attributable to physical redistribution of the drug rather than to chemical destruction, as intraperitoneal doses of 1.5 g per kg will maintain deep anesthesia in mice for about 2 hours. It would appear not unlikely that the rate of elimination of DMO may be slow relative to the rate of its production from trimethadione. In this circumstance the chronic oral administration of trimethadione would lead to more extensive accumulation of the product of demethylation than of the original drug, as has been demonstrated in the analogous case of mephobarbital (N-methyl phenobarbital) (3). Knowledge of the actual extent of accumulation of DMO resulting from trimethadi-

one administration will be possible only when an analytical method is available for the determination of DMO in blood. In the treatment of epilepsy with trimethadione it may well be that DMO plays a significant part in the therapeutic effect.

Summary. 3,5,5-Trimethyl-2,4-oxazolidinedione (trimethadione, Tridione) is demethylated by the dog to yield 5,5-dimethyl-2,4-oxazolidinedione. This metabolic product has been isolated from urine and identified.

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Application of Histochemical Technic for Cholinesterase to Paraffin Sections.* (19908)

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(Introduced by J. S. Nicholas.)
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The application of histochemical technics for enzyme localization in tissue sections offers one a promising approach to the problem of biochemical differentiation. In embryological work, however, it is often necessary to examine the material not on random and isolated sections, but on serial sections from which spatial relationships of the various structural elements can be reconstructed. For the preparation of serial sections, the paraffin method is at present the only convenient and reliable technic. Unfortunately not all enzymes survive the harsh treatment usually involved. The enzyme cholinesterase, in which we have been particularly interested in relation to neural differentiation, is one which is relatively susceptible to inactivation. Gomori's method(1) for cholinesterase localization was devised for use with paraffin sections, but the extremely low rate of reaction and the non-specificity of the substrates (long-chain fatty acid esters of choline) restrict its usefulness. In the histochemical technic for cholinesterase recently developed by Koelle and Friedenwald(2) and Koelle(3), the substrate used was acetylthiocholine which was found to be hydrolyzed by the enzyme at a rate comparable to that of the natural substrate, acetylcholine. Furthermore, by the use of appropriate concentrations of DFP, this technic is capable of differentiating the 2 types of cholinesterase. The method was used only for fresh teased preparations or frozen sections. To increase the usefulness of this technic, particularly for embryological work, we attempted to explore the possibility of preparing the specimen for paraffin sectioning with a minimum loss of enzyme activity prior to histochemical treatment.

The enzyme content of normal frog brain has shown a high degree of bilateral symmetry. It is, therefore, ideally suited for the present work of testing the effect of various histological treatments on the enzyme activity of the tissue. The brain was divided into left and right halves, one serving as control. Since the frog central nervous system has been shown to contain exclusively "specific" cholinesterase, all enzyme activities were measured manometrically using acetylcholine as substrate.

Effect of fixatives and lyophilization on cholinesterase activity. It is obvious that for histochemical purposes, the fixatives usually employed in histological procedures should be avoided. Although acetone has been most commonly used as a fixative in histochemical applications, prolonged treatment of the fresh tissue with cold acetone was found to inactivate most of the cholinesterase. Ethyl al-

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TABLE I. Effect of Fixing and Dehydration on Cholinesterase Activity of Fresh Tissue.

Treatment	Cholinesterase activity		% inactivation
	Untreated	Treated	
Ethyl alcohol (½ hr 0°C)	336	0	100
Acetone (½ hr 0°C)	392	107	73
Freeze-drying	385	387	0

cohol completely destroyed the enzyme (Table I). As an alternative to the use of fixative and dehydrating reagents, fresh tissue was lyophilized in preparation for paraffin embedding. For practical purposes we found Gersh's technic for freeze-drying too cumbersome, and the time required for complete dehydration excessively long. A greatly simplified version of the technic was therefore adopted. The tissue was frozen in acetone previously cooled in a stainless-steel vessel immersed in a freezing mixture of acetone and solid CO_2. The frozen tissue was transferred with a pre-chilled spatula into the lyophilizing vessel kept at low temperature. Once high vacuum was established, no attempt was made to regulate the temperature of the drying tissue. No thawing occurred throughout the process of lyophilizing under these conditions. The average time for complete dehydration of tissue the size of the adult frog brain was less than one hour. Smaller fragments of tissue were dehydrated in considerably less time. The cholinesterase activities were determined on the homogenates of the treated and untreated brain halves. For comparison, the activities are expressed in arbitrary units per half-brain. It is clear that the enzyme activity of fresh tissue is completely unaffected by the present procedure of lyophilizing.

Effect of embedding, sectioning and hydrating. During embedding, temperature inactivation of the enzyme was minimized by the use of low melting-point paraffin (Bioloid, 43-45°C) and brief period of infiltration. The lyophilized tissue was introduced directly into a vessel of melted paraffin kept at a constant temperature of 45°C with immediate evacuation of the vessel continued for one minute. Upon breaking the vacuum the tissue sank to the bottom and was then embedded in the usual manner. Thus the total time in which the tissue was exposed to 45°C was less than 3 minutes. Subsequent sectioning showed that infiltration was complete. Sections were usually cut at 20-40 μ. For thinner sections, it would be necessary to employ some means of chilling the paraffin block as well as the knife. Since the tissue had not been fixed, the use of water as a flotant had to be avoided in mounting the sections. The sections were affixed directly upon albuminized slides and flattened by pressing with a camel's hair brush while warming the slide by the heat of the hand. Since no drying was necessary, the slides could be deparaffinized immediately in xylol which does not affect the enzyme activity. Experiments were then carried out to ascertain the most suitable means of hydrating the deparaffinized sections. The effect of the commonly used hydrating agents on enzyme activity was determined in the following manner. The mounted sections of an entire half-brain were deparaffinized in xylol, and treated with various hydrating agents. After passing into aqueous medium, they were care-

TABLE II. Effect of Hydrating Agents on Cholinesterase Activity of Tissue Sections.

Material	Hydrating agent	Cholinesterase activity		% inactivation
		Untreated	Treated	
Frog brain	None (sections dried in air)	356	348	2
" "	Dioxane	375	0	100
" "	Alcohol	324	0	100
" "	Acetone	432	333	22
Rat ventricle*	"	158	116	26
" " †	"	51	35	31

* Acetylcholine-hydrolyzing activity—μl CO_2/mg N/hr.
† Benzoylcholine- " " " .

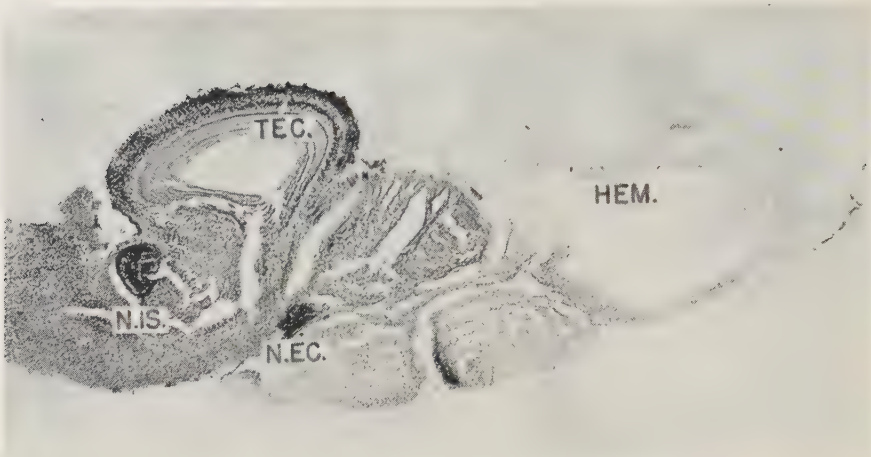

FIG. 1. Histochemical demonstration of cholinesterase in frog brain. Sagittal section. CH., optic chiasma; HEM., hemisphere; N.EC., nucleus ectomamillaris; N.IS., nucleus isthmus; TEC., tectum.

fully removed by scraping from the slide, avoiding any loss of material, and then homogenized in Ringer-bicarbonate solution. The activity of the treated half was measured and compared with that of the untreated lyophilized half of the brain. Cholinesterase activities are expressed in arbitrary units per half-brain. Average values from a series of experiments are given below.

As shown above, the use of alcohol or dioxane quickly and completely inactivates the enzyme. On the other hand, relatively little damage to the enzyme was caused by similarly brief treatment with acetone, although prolonged exposure to acetone resulted in extensive inactivation of the enzyme (Table I). Similar results were obtained with rat ventricles which contain predominantly non-specific cholinesterase. The degrees of inactivation of the 2 cholinesterase types are comparable.

Histochemical results. The histochemical technic employed in the present investigation is that of Koelle(3). The deparaffinized sections, after 2 brief rinsings in cold acetone and in Ringer's solution, were transferred directly to the incubation solution. Half an hour of incubation at 37°C was found to be adequate for frog brain. After the development of color in H_2S, the sections were fixed in 10% formalin saturated with CuS. They were then dehydrated through CuS-saturated alcohols, cleared in xylol and mounted in balsam. A typical picture of cholinesterase distribution in adult fog brain is shown in Fig. 1. The almost complete absence of staining of the hemisphere will be noted, as well as the concentration of the enzyme in the tectal layer of the optic lobe and some of the neurological centers, notably the nucleus isthmus. Detailed description of the enzyme distribution in frog brain will be given elsewhere. Brief incubation of the sections in 10^{-5} M DFP prior to the addition of substrate completely inhibited the histochemical staining. Cytologically, the enzyme activity seems to be concentrated in or on the nuclei. This may, however, be a diffusion artefact as suggested by Koelle(4) in his later modification of his technic, in which it was shown that if intracellular diffusion of the enzyme were controlled by means of high salt concentration at a lower pH, the site of enzyme activity was localized in the cytoplasm. Unfortunately, under these conditions, the enzyme activity was found to be greatly depressed, especially that of specific cholinesterase which was reduced by as much as 80%. When this modified histochemical procedure was applied to paraffin embedded and sectioned material, the staining was found to be feeble and poorly differentiated even after prolonged incubation due presumably to an excessively high overall inactivation of the enzyme. The use of unphysiologically high concentrations of salt in

the reaction mixture as a means of "fixing" the enzyme raises the question of the *normal* state of diffusibility of the enzyme within the cellular structure. In any case, the exact cytological localization of cholinesterase in nerve cells cannot at present be considered unequivocally established. For the demonstration of the site of enzyme activity on a histological level, the present adaptation of the original histochemical procedure in which intracellular diffusion of the enzyme is not "controlled," seems entirely adequate and satisfactory.

It should be admitted that the very much simplified and abbreviated procedure of tissue lyophilization used here does result in some minor imperfections in the tissue sections, especially when the tissue is excessively bulky, *e.g.*, whole brain of an adult frog. Improved quality of the sections could, however, be obtained by the employment of more elaborate apparatus for critical temperature regulation in the process of lyophilization.

Summary. The histochemical technic of Koelle for cholinesterase demonstration in fresh tissue was adapted to paraffin sections. The effect of certain histological treatments on enzyme activity was determined. A procedure is described for the preparation of the specimen for paraffin sectioning prior to histochemical treatment with maximum retention of enzyme activity.

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Production of Tolerance in the Rat to Octamethyl Pyrophosphoramide (OMPA).* (19909)

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Octamethyl pyrophosphoramide, first synthesized by Schrader(1) in 1947, has become of increasing interest as a systemic insecticide† as indicated by the reports from England of Ripper, *et al.*(2) and Heath, *et al.*(3). Following an extensive pharmacological study by DuBois, Doull and Coon(4), in which it was demonstrated that this compound is a potent *in vivo* anticholinesterase agent with a selective action on the cholinesterase of peripheral tissues and that its toxicity could be effectively reduced with atropine (increasing the LD_{50} for mice and dogs fourfold), it aroused considerable interest as a possible therapeutic agent in the treatment of myasthenia gravis. For this purpose it has been used with success by at least two different groups of investigators in the United States, namely, Rider, *et al.*(5) and Gregory, Futch and Stone(6). In addition to this use it is also now available as an insecticide (Monsanto Chemical Co.). Since the therapeutic use of OMPA in myasthenia gravis involves chronic administration, and since its manufacture and use as an insecticide involve probable repeated exposures it is highly desirable to obtain as much information as possible about the toxicity of this compound. Of special concern is knowledge of the cumulative effect of daily administration of the drug, tolerance to the drug, and possible long range pathologic effects. DuBois, Doull

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† A systemic insecticide is defined as one that is absorbed by the plant and translocated in the sap so that parts of the plant other than those treated become toxic to (sucking) insects.

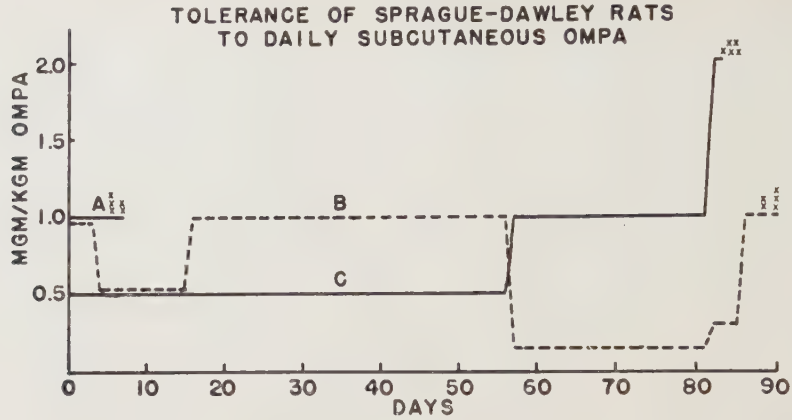

FIG. 1. Graphic representation of the OMPA dosage schedules in different groups of rats. The dose, as indicated on the ordinate, was given daily for the number of days indicated on the abscissa. Each × represents the death of 1 rat, and the number of ×'s on each line represents the total number of rats started in the corresponding group.

and Coon(4) have previously shown that the LD_{50} for parenterally administered OMPA in rats was about 8 mg‡ when given in one dose. They also showed that the daily administration of 2 mg caused death in 50% of the animals after the third dose, and the remainder of the animals died after the fourth dose. The failure of at least some of the animals to survive more than a total of one single-dose LD_{50} given at the rate of 2 mg/day was unexpected. Daily doses of 1 and 1.5 mg had a similar effect. Thus with these daily doses an efficient cumulation was observed and death of the animals occurred when the total quantity of OMPA administered was close to the LD_{50} value obtained for single doses. In this same experiment, however, it was reported that the animals tolerated daily doses of 0.25 and 0.50 mg/day for as long as 60 days, indicating a lack of efficient cumulation at these doses or suggesting the possibility that some degree of tolerance may develop.

The following experiments were undertaken in order to determine whether any tolerance would develop in rats that were treated daily with sublethal doses of OMPA.

Materials and methods. Adult male Sprague-Dawley or Maguran rats were employed as the test animals. OMPA was administered subcutaneously as a 0.1% aqueous solution. Cholinesterase measurements were performed manometrically essentially according to the method described by DuBois and Mangun(7). Blood was obtained for the assays from the tails of the rats in 0.2 cc amounts. After the Warburg flasks were gassed for 5 minutes with 95% N_2 and 5% CO_2 and equilibrated for 15 minutes, the acetylcholine was tipped in from the side arm. Readings were taken at 5-minute intervals for 30 minutes. The activity of the whole blood cholinesterase (representing a combination of the red cell and serum cholinesterase) was expressed in microliters of CO_2 liberated in an average 5 minute period.

Results. In Fig. 1 is illustrated the OMPA dosage schedules and mortality results (each x = 1 death) of three groups of five Sprague-Dawley rats each. Group A received 1 mg daily. Three of these animals died after the sixth dose and the remaining two died after the seventh dose. Group B received 1 mg daily for three days. The dose was then decreased to 0.5 mg for 12 days. Following this it was increased to 1 mg for 41 days. During this period, in contrast to the results in group A, there were no deaths, nor were there any toxic symptoms. Next, the daily dose of these animals was reduced to 0.15 mg for 25 days, increased to 0.3 mg for 4 days and finally to 1 mg. This latter dose caused death in two of the animals after the fourth dose and in the remaining three after the fifth dose. Thus the tolerance that had previously developed in this group was lost after a pro-

‡ All doses given in this paper are expressed in terms of body weight in kilograms.

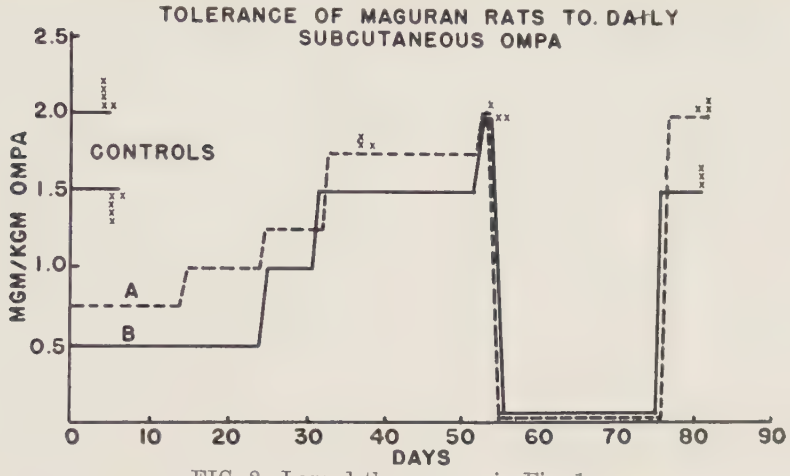

FIG. 2. Legend the same as in Fig. 1.

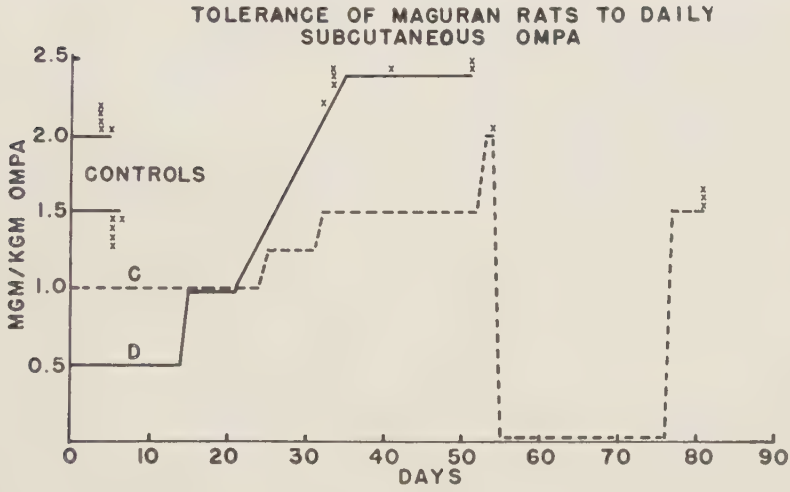

FIG. 3. Legend the same as in Fig. 1.

longed period of very low dosage followed by a sudden return to the previously tolerated dose. Group C was given 0.5 mg for 56 days. This was increased to 1 mg/day for 26 days without fatality. Two mg for 2 days, however, resulted in 100% mortality; thus Group C also demonstrated the ability to tolerate 1 mg for a long time. These experiments show that a tolerance to a 1 mg/day schedule can be induced by a daily dose of 0.5 mg given for 12 or more days.

Dosage schedules and mortality results for different groups of Maguran rats are graphed in Fig. 2 and 3. Although this strain seemed to be more resistant to the OMPA than the Sprague-Dawley, the results in general were in conformity. A daily dose of 2 mg caused 100% mortality in 5 days and a daily dose of 1.5 mg caused 100% mortality in 6 days. These groups of 5 rats each are considered to be the controls in both Fig. 2 and 3.

Group A (Fig. 2), consisting of 7 rats received 0.75 mg for 14 days, then the dose was gradually increased. Four of the rats tolerated 1.75 mg for 20 days but could not tolerate 2 mg. Group B was treated in a similar manner. Whereas they could tolerate 1.5 mg for 21 days they lost this ability after a prolonged rest period. Group C (Fig. 3) was begun at 1 mg for 24 days and the results were similar to those observed in Group B. After group D received 1 mg for 7 days the

TABLE I. Cholinesterase Inhibition by OMPA of Maguran Rat Whole Blood.
(See Fig. 2 and 3 for dosage schedules.)

Group	Control	22 days	36 days	21 days rest
A		7.1	8.8	
B		5.1	6	
	21.8*			21.7
C		4.4	6.8	
D		5.3	6	

* μl CO_2/5 min./.2 cc.
Control and 21 days rest values are from pooled blood samples from individuals of each group.

dose was increased by increments of 0.1 mg/day for 14 days. One rat died after it had received the dose of 2.2 mg and 3 died after they had received 2.3 mg. One rat tolerated 2.4 mg for 7 days and the remaining 2 rats did not die until the 17th day.

The results obtained from the Maguran rats indicate that whereas 1.5 mg of OMPA daily caused 100% mortality of the rats so treated for 6 days, it was possible, by means of slowly increasing the dose of OMPA, to build up a relative tolerance to a daily dose as high as 2.2 to 2.4 mg, although at this latter dose all rats were dead after 17 days.

Additional suggestive evidence for tolerance can be seen in Table I in which the cholinesterase inhibition by OMPA of Maguran rat whole blood is shown. It can be seen that the levels of cholinesterase activity in the various groups are about the same on the 22nd and 36th day of treatment, with a slight tendency to increase on the latter day. After 21 days without treatment the blood levels had returned to pre-treatment values.

Although all the rats before death had typical cholinergic symptoms, such as diarrhea, exophthalmos, muscular twitchings, salivation, and respiratory distress indicative of bronchial spasm, the gross and microscopic examinations of 14 of the rats shortly after death revealed no abnormalities in the liver, kidney, intestine, heart, spleen, skeletal muscles, lungs or brain.§ It was concluded that death was due to biochemical causes with no change in morphology.

Discussion. The results presented here indicate that it is possible to increase the tolerance of rats to OMPA by gradually increasing the daily dose. The most striking characteristic of this tolerance is not the size of the dose of OMPA which could be tolerated, but rather the length of time that the animals could tolerate, without symptoms, a daily dose which normally killed in a few days. This tolerance was lost after a period of discontinued administration of the OMPA. Blood cholinesterase levels were in accord with the above observations since the levels did not continue to decrease after prolonged treatment and even tended to show a slight rise.

The mechanism by which this tolerance develops is unknown. It is possible that a) production of cholinesterase has been stimulated, b) detoxification mechanisms are more rapid, or c) the conversion of OMPA to an active cholinesterase inhibitor(4) by the liver is slower. Since the mechanism of this increased tolerance is unknown we have little information upon which to base the importance of these findings in connection with the daily use of OMPA in myasthenia gravis patients or in chronic exposure in those who are using this agent as an insecticide. If man also develops a tolerance, however, it would appear wise to increase a patient's dose of OMPA gradually in order to induce a tolerance which would be expected to minimize the incidence of toxic reactions. Furthermore, after the administration has been stopped for any length of time it might be hazardous to restart it at the same dose level as previously tolerated. One might also expect less toxic effects after prolonged administration of the same dose. Although myasthenia gravis is a variable disease we have seen some clinical evidence of a tolerance developing to OMPA. For example, we have seen patients who, while on the same daily dose of OMPA will no longer need atropine to counteract undesirable cholinergic symptoms after several weeks of therapy.

Summary. The cumulative effect of OMPA in rats has been confirmed. It was possible to induce a tolerance to OMPA by gradually increasing the daily dose or by treating the animals daily with a sublethal dose for a pro-

§ Grateful acknowledgement is made to Dr. John B. Storer for the pathologic studies.

longed period. Animals prepared in this manner were able to tolerate for several weeks, without symptoms, a daily dose which would normally kill all rats in a few days. No gross or microscopic pathological changes were seen in the organs of the rats examined in this study.

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In vivo Inhibition of Liver and Brain Monoamine Oxidase by 1-Isonicotinyl-2-Isopropyl Hydrazine.* (19910)

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It has been shown that 1-isonicotinyl-2-isopropyl hydrazine (IIH) inhibits *in vitro* the monoamine oxidase of liver and brain of 6 different species(1,2). The inhibition is marked at concentrations as low as 0.2×10^{-5} molar. The question thus arose as to the possible occurrence of such a reaction *in vivo,* with the consequent production of symptoms as observed in the therapeutic application of IIH.

Studies on the cellular localization of monoamine oxidase reveal the enzyme activity to be largely present in the mitochondria(3-5). The addition *in vitro* of IIH to a mitochondrial suspension results in the disappearance of the monoamine oxidase activity. There is no restoration of activity by dialysis for 3 hours. It seemed then, that the administration of IIH to intact animals, followed by the isolation of mitochondria from tissues for the determination of monoamine oxidase activity would provide an indication as to whether the enzyme is inhibited *in vivo.*

Procedure. 1-Isonicotinyl-2-isopropyl hydrazine phosphate (IIH)† and isonicotinic acid hydrazide (INH)† dissolved in M/15 phosphate buffer pH 7.2 were injected subcutaneously into adult, male and female rats (Sprague-Dawley) and guinea pigs. Two hours later the animals were sacrificed by decapitation or by intravenous injection of air. The brain and liver were immediately removed, washed and chilled. Mitochondria were isolated by centrifugal fractionation of 1:10 tissue homogenates in 0.88 M sucrose solution according to the method of Hogeboom *et al.*(6). The characteristic morphology of the mitochondria was confirmed by phase microscopy. The determination of monoamine oxidase activity was carried out by the conventional manometric procedure for the estimation of oxygen uptake, and by microdiffusion analysis for the estimation of ammonia production. The incubation system contained, in a final volume of 2 ml, homogenate or washed mitochondria suspended in M/15 phosphate buffer pH 7.2 and 0.01 M tyramine hydrochloride (F. Hoffmann-La Roche); oxygen was employed in the gas phase. The enzyme suspensions per vessel were equivalent to 0.1 g fresh tissue for liver homogenate, 0.2 g original tissue for liver mitochondria and 0.33 g grey cortical tissue for brain mitochondria. The incubation was

* This work has been aided by a grant from the Multiple Sclerosis Foundation of Illinois.

† The compounds used were obtained from Hoffmann-La Roche, Inc., Nutley, N. J. IIH = Marsilid, INH = Rimifon, and from Bristol Laboratories, Syracuse, N. Y. (INH).

TABLE I. Effect of *In Vivo* Administration of Isonicotinic Acid Hydrazide (INH) and 1-Isonicotinyl-2-Isopropyl-Hydrazine (IIH) on Monoamine Oxidase of Rats.

Inhibitor, mg	Oxygen uptake, μmoles	Ammonia production, μmoles
	Liver homogenate	
0	90.5	119
IIH 10*	0	6
IIH 20	0	6
INH 10†	95.5	115
	Liver mitochondria	
0	34	40
IIH 10	1	1.5
IIH 20	3.3	2
INH 10	35.8	41
	Brain mitochondria	
0	12.9	14.4
IIH 10	4.8	4.8
IIH 20	1.5	1.5
INH 10	13.9	14.6

* 10 mg IIH = .036 millimole/rat = to .1 millimole/kg body wt.

† 10 mg INH = .073 millimole/rat = to .29 millimole/kg body wt.

TABLE II. *In Vitro* Effect of Hydrazides on Monoamine Oxidase of Guinea Pig Liver Mitochondria.

Inhibitor	Oxygen uptake, μmoles	Ammonia production, μmoles
0	46	53.5
.1 millimolar IIH	20.5	23
1 " INH	45.5	59.5

TABLE III. Effect of *In Vivo* Administration of 1-Isonicotinyl-2-Isopropyl-Hydrazine on Monoamine Oxidase of Guinea Pigs.

Inhibitor, mg	Oxygen uptake, μmoles	Ammonia production, μmoles
	Liver homogenate	
0	116	125
IIH 25*	7	4
	Liver mitochondria	
0	43.3	52
IIH 25	.5	1
	Brain mitochondria	
0	6.9	8.7
IIH 25	.9	1.2

* Equivalent to .18 millimole/kg body wt.

carried out at 38°C for 2 hours, after which 1 ml of the reaction mixture was transferred to Conway diffusion vessels. For final values, blank determinations were subtracted from those for the reaction mixture. The results are presented in the form of values per g fresh tissue for the 2-hour incubation period. Each set of values represents a mean of determinations on 2 animals.

Results. The administration of IIH leads to a nearly complete disappearance of monoamine oxidase activity of liver homogenate and mitochondria, and of brain mitochondria. INH, which even at 10^{-3} molar concentration *in vitro* has slight or no effect on liver(1) and brain monoamine oxidase(2) of the rat, also produces no apparent inhibition of the enzyme when administered *in vivo* (Table I).

Since no data on the *in vitro* effects of IIH and INH on guinea pig monoamine oxidase have been reported previously, typical values are presented in Table II.

The mitochondrial monoamine oxidase of guinea pig liver seems to be less sensitive toward IIH than that of the rat, which under identical conditions, is more completely inhibited. Nevertheless, after injection of IIH into guinea pigs, the enzyme activity again disappeared (Table III).

Discussion. Inasmuch as monoamine oxidase activity disappears from homogenates as well as from mitochondrial preparations, it is unlikely that the loss from mitochondria could be attributed to a change of the distribution of the enzyme among the different cell components, but points rather to an inactivation of the enzyme "in loco" by IIH. The observations indicate, therefore, that IIH not only enters the cell but also penetrates the mitochondria.

The elimination of monoamine oxidase activity *in vivo* should prove of value in the investigation of the physiological function of the enzyme, particularly with regard to its possible role in the degradation of epinephrine and nor-epinephrine. IIH may well become of the same importance for the study of the adrenergic system as has eserine in the analysis of the cholinergic system. Significantly, both types of brain cholinesterase are uninfluenced by the hydrazides(2). The above results may also offer an explanation for the sympathetic and general mental stimulation observed after administration of iso-

nicotinyl-hydrazides to patients and animals (7), in that the blocking of monoamine oxidase activity could conceivably lead to an accumulation of sympathomimetic amines with ensuing effects on the autonomic nervous system.

Summary. After the subcutaneous injection of 1-isonicotinyl-2-isopropyl hydrazine (IIH) into rats and guinea pigs, the monoamine oxidase activity of liver homogenates, liver mitochondria, and brain mitochondria is almost completely inhibited. The probable significance of such an inhibition is indicated.

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Dual Infection of Dogs with Distemper Virus and Virus of Infectious Canine Hepatitis.* (19911)

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Comparative studies(1) have shown that the illness produced by distemper virus resembles clinically the illness produced by the virus of infectious canine hepatitis. Both diseases were believed originally to be only one disease which was caused by the distemper virus, one of the earliest viruses isolated(2). Recent recognition of infectious hepatitis as a clinical entity(3) caused by a specific virus that produces characteristic intranuclear inclusion bodies, especially in hepatic cells, has now made possible the differentiation between this entity and that of distemper by histological studies. Shortly after the virus of infectious hepatitis was reported, mention was made of finding inclusion bodies of both distemper and infectious hepatitis in a dog that died from an illness attributed to distemper virus(4). Both distemper and infectious hepatitis have a high incidence rate(3,5). Following the report of inclusion bodies of both viruses in the same dog, further clarification seemed desirable. Studies were, therefore, undertaken in order to determine whether the two distinct viruses could infect dogs simultaneously, whether there would be discernible interference, and what the effects might be of such a concurrent infection. The results are reported herewith.

Experimental infection in dogs. Production. Puppies from 9 to 16 weeks of age were used and were obtained in litters of 3 or more. Prior to inoculation each litter was placed in an isolation unit and observed for at least a week for any sign of illness. During this period, temperatures and total leukocyte counts were taken daily. In instances where any puppy of a litter showed a temperature exceeding 39.5°C or a leukocyte count that deviated from the normal range, the entire litter was rejected. After the initial period of observation, puppies of each litter were divided into 3 groups and each group was placed in a separate isolation unit before inoculation. Each dog in 1 group was inoculated intravenously with 1 ml of a suspension in saline of spleen from a dog that had been given a strain of distemper virus originally obtained

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TABLE I. Comparison of Effects Produced in Dogs by Infectious Canine Hepatitis Virus (IH) and Distemper Virus (D) Singly and Mixed.

Virus	No. of dogs	Results of inoculation: Day of fever onset 1	2	3	4	5	Day died after inoc. 3	4	5	6	7	8	16+*	% died
IH	12		7	5				1	2	1	2	1		58
D	12		5	7									2	17
D and IH	28	26	2				12	4	4	1			1	79
D followed by IH	9	4	4	1			3	1	3	1				89
IH followed by D	5			4		1								0

* + means died 16 days or more after inoculation.

from the Fromm Laboratories. Each dog in the second group was inoculated intravenously with 1 ml of a suspension in saline of spleen from a dog that had been given Cornell Strain I infectious canine hepatitis virus, and each dog in the third group was inoculated with 2 ml of a mixture of equal parts of the same viral suspensions that were used for the other groups. Observations, temperatures and total leukocyte counts were recorded daily. These tests were repeated on 11 litters that represented 12 dogs in each group that received a single virus and 28 dogs the mixture. The results are presented in Table I.

Signs of illness. As can be seen in Table I, simultaneous infection with the viruses of infectious canine hepatitis and canine distemper produced a more severe illness in dogs than did either virus alone. In 26 of 28 dogs the incubation period was shortened from 2 days to 1 day. The percentage of deaths was higher, increasing from 17% for distemper and 58% for infectious hepatitis to 79% for both, and deaths resulted more quickly. In addition, the temperature and leukopenic reactions were more marked and the convalescent period of survivors was longer.

Presence of viruses in blood. From dogs that had been given both viruses at the same time, blood was obtained by venipuncture on the second day of fever, placed in sterile vials and stored under dry ice refrigeration. Then each of the 5 dogs that had survived inoculation with infectious hepatitis virus and subsequently shown to be immune to infectious hepatitis virus by a second inoculation was given intravenously 1 ml of the stored blood from a different dog that had been inoculated simultaneously. In like manner, 5 of the 9 dogs that had recovered from distemper and were shown to be immune by a second inoculation of distemper virus were each given blood from the same dogs that had furnished inoculation for those immune to infectious hepatitis. In addition, the other 4 dogs from the distemper-recovered group were given blood obtained from 4 other dogs that had been given the mixture of viruses. These results are also included in Table I. In all cases, dogs became ill and showed clinical signs referable to the respective viruses. That is, dogs that were immune to infectious hepatitis at the time of test developed distemper, whereas those immune to distemper developed infectious hepatitis. Blood from dogs given the mixture, therefore, contained both viruses during the febrile period.

Dogs immune to infectious hepatitis and then given the infective blood from dogs inoculated with the viral mixture developed an illness which resembled the disease in those given distemper virus alone. Dogs recovered from distemper and then given the infective blood, however, showed an illness which resembled that of the dual infection. Onset of fever was more rapid, the survival period was shortened, and the mortality rate was higher.

Presence of inclusion bodies. From 5 dogs that died, 3 on the third, 1 on the fourth and 1 on the fifth day after inoculation, thin slices of liver, lung and bladder were fixed in Zenker's, stained by Wilhite's method and examined for inclusion bodies of distemper and of infectious hepatitis. Typical intranuclear inclusion bodies of infectious hepatitis were found in hepatic cells of all dogs. One of the dogs that died 3 days after inoculation showed a few indistinct inclusions of distemper while the other 2 did not. The dog that died on the fourth day and the one on the fifth day

showed numerous well defined inclusion bodies of distemper.

Immunity. In tests for immunity, each of 5 animals that survived initial inoculation of the viral mixture was given intravenously, after 30 days, 1 ml of a suspension of single virus, followed in 21 days by a similar inoculation of the other virus. After inoculation, each animal was carefully observed, temperatures taken and leukocyte counts made. None of these animals showed signs of illness from inoculation with either infectious hepatitis virus or distemper virus. This indicated that immunity had developed against both of these diseases from the viral mixture.

Discussion. Following inoculation of infectious canine hepatitis virus and distemper virus simultaneously into dogs, both viruses were present in the blood during the acute phase of illness, inclusion bodies typical of distemper and infectious hepatitis virus were found in dogs that died, and immunity developed against both viruses. The conclusion was reached that dual infection had been produced in dogs.

A comparison of clinical signs shown by dogs that had received the mixture of viruses with those produced by each virus singly showed that signs of illness were more severe and a higher mortality rate was produced by the mixture. This finding may explain certain fatalities heretofore attributed to either distemper or infectious hepatitis, especially in instances where distemper virus is the precedent infection and thereby enhances the effects produced by infectious hepatitis.

The whole subject of dual infections or its converse, interference, presents many interesting aspects(6,7) particularly when related to the subject of immunization. Obviously, combinations of 2 or more viruses that produce dual or multiple infections simultaneously can permit combined vaccination procedures. In the case of infectious hepatitis and distemper, Poppensiek(8) has shown that dogs can be immunized consistently by a mixture of both viruses.

Summary. Following inoculation of distemper virus and infectious canine hepatitis virus singly and mixed, it was found that the mixture of viruses produced a more severe type of illness than either virus alone. In the same dog, both viruses were present in the blood during the acute phase of illness, inclusion bodies of both viruses were found and immunity against both viruses developed.

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Antitussive Action of *d*-Isomethadone and *d*-Methadone in Dogs. (19912)

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In the management of unproductive or useless cough (tussal insufficiency), potent analgesic drugs are generally employed. Among the active cough suppressants which have been used for this purpose are morphine, ethyl-morphine, codeine, dihydrocodeinone, and methadone. In addition to their antitussive action, these drugs have a general hypnotic effect, they are respiratory depressants, they are often nauseant, they produce constipation, tolerance develops to their use, and above all they possess addiction liabilities.

With the exception of the recent report of Toner and Macko(1), on experiments per-

TABLE I. Suppression of Cough in 11 Dogs by Orally Administered Codeine.

Dog No.	Drug	Dose, mg/kg	No. of coughs/5 min. Before drug Hr 2	Hr 1	Hr 0	After drug Hr .5	Hr 1	Hr 2	Hr 3
1	None (controls)	—	—	—	40	—	47	38	—
2		—	—	—	14	—	10	12	—
3		—	—	—	34	—	55	46	—
4		—	—	—	42	—	47	56	—
5		—	—	—	18	—	29	21	—
6	Codeine phosphate	.25	—	30	28	25	32	28	—
7		.25	—	19	19	18	2	3	14
8		.5	12	16	15	—	3	12	—
9		.5	—	21	19	—	10	13	—
10		.5	28	26	28	9	6	18	—
11		1	17	21	23	0	1	9	24

formed on anesthetized cats, objective evidence is lacking that potent antitussive activity is possessed by compounds having little or no narcotic or analgesic effect. This communication presents such evidence in unanesthetized dogs. It is generally known that the analgesic and narcotic activities of *dl*-isomethadone and *dl*-methadone reside mainly in the *levo* isomer, the *dextro* isomers being relatively free of these effects. We have obtained evidence, however, that *d*-isomethadone and *d*-methadone possess an appreciable degree of antitussive activity.

Materials and methods. For the production of cough, dogs were placed in a wood and glass box about 56 cm square and 42 cm high. Through 2 holes, a fine aerosol mist of N/2 H_2SO_4 was sprayed into the box by means of 2 Vaponephrin nebulizers operated by compressed air at a pressure of about 400 mm Hg. Many animals have been exposed repeatedly without any signs of ill effects. A count of coughs during a 5-minute exposure was obtained by attaching a throat microphone to the animal, the observer listening by means of headphones. Drugs were administered either in dry form in gelatin capsules, or (more usually) in aqueous solution by stomach tube following an 18-hour food fast. In some experiments, drugs were injected subcutaneously. At least 2 control readings an hour apart were taken before administration of drugs, and subsequent counts were made at appropriate intervals after the drug. About one week rest was allowed for each animal between experiments. In no instance has either tolerance or increased sensitivity either to the procedure or to the drugs been observed. This method is a modification of that used in guinea pigs by Eichler and Smiatek(2).

Results. The consistency of the number of counts obtained from an animal not receiving an antitussive agent, and repeatedly exposed in the chamber, is shown in Table I. The data also demonstrate the responsiveness of the preparation to a known antitussive agent. The number of coughs was markedly reduced in one of 2 animals receiving 0.25 mg per kg of codeine phosphate, and in all those receiving higher doses.

The data in Table II show that both *d*-isomethadone and *d*-methadone produced a degree of cough suppression comparable to that produced by codeine, and the effective doses of the former drugs were only slightly higher than those of codeine. We have been unable to demonstrate a significant degree of analgesia or hypnosis when *d*-isomethadone was tested in rats in doses up to 64 mg per kg subcutaneously or 128 mg per kg orally by a method previously described(3). Others have also found that such effects are minimal in *d*-isomethadone and *d*-methadone, although the *levo* isomers are potent analgesics(4-7). Four experiments with 1-isomethadone are included in Table II, for purposes of comparison. According to Eddy *et al.*(7), *l*-isomethadone is about 40 times as potent an analgesic as is the *d* isomer. The data in Table II indicate that it is no more than 4 times as potent an antitussive. It is thus clear that the

TABLE II. Suppression of Cough in Dogs by Orally Administered *d*-isomethadone and *d*-methadone.

Dog No.	Drug	Dose, mg/kg	No. of coughs/5 min. Before drug Hr 1	Hr 0	After drug Hr .5	Hr 1	Hr 2	Hr 3
12	*d*-isomethadone HCl	.5	28	25	16	18	28	—
13		.5	19	24	13	19	26	—
14		1	56	52	13	14	57	—
15		1	18	24	5	16	18	—
16		1	22	34	9	0	18	24
17		1	19	19	5	2	23	17
16		2	31	29	27	11	7	9
18		2	17	17	0	2	0	19
9		2	15	26	23	2	4	3
6		2	14	19	0	0	17	26
19	*d*-methadone HCl	.5	23	15	6	6	12	14
12		.5	28	30	21	25	20	27
16		.5	18	23	19	25	28	—
7		1	14	21	7	5	17	14
18		1	15	19	5	3	11	5
18	*l*-isomethadone HCl	.25	19	17	0	14	21	20
7		.25	26	29	15	19	24	23
17		.5	23	26	0	0	26	20
16		.5	28	31	0	10	13	24

antitussive activity is not dependent upon analgesic or narcotic effects.

An unexpected finding was that *d*-isomethadone is much less effective as an antitussive agent in dogs when administered subcutaneously than when given by mouth. The following experiment was performed: 6 dogs were selected; 2 received each of the following doses subcutaneously: 1, 2, and 10 mg/kg. The 2 dogs on the highest dose showed nearly complete suppression of coughing, but the other 4 showed no effect within 2 hours. The first 2 were then given 2 mg/kg, and the second 2, 1 mg/kg by stomach tube. Two of these 4 responded by complete or nearly complete suppression of coughing, while the other 2 had about 50% suppression within an hour. Two dogs which received 1 mg/kg of codeine phosphate subcutaneously gave only a moderate response. Although we have not made a detailed comparison of the effectiveness of oral and subcutaneous codeine in dogs, the preliminary indication is that codeine also may be more effective by the former route.

Summary. A method is described for testing antitussive activity in dogs. It is shown that 2 compounds nearly devoid of analgesic or narcotic properties, *d*-methadone and *d*-isomethadone, are active in this test.

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Resistance of Normal Human Thyroids to Propylthiouracil.*† (19913)

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Propylthiouracil in large doses has been administered to 8 euthyroid women in an attempt to induce hypothyroidism and the menstrual abnormalities associated with the hypothyroid state. Although the therapy was continued for 7½ months and the dosage for the last 6 weeks was 3000 mg daily in divided portions, no instances of hypothyroidism or myxedema occurred.

Since thiouracil and thiouracil derivatives are so effective in controlling the hyperactive thyroid, there have been repeated attempts to decrease the activity of the normal thyroid in angina patients where a general decrease in metabolism is desired. Table I recapitulates the literature available on the effectiveness of thiouracil on euthyroid individuals. An interpretation of the table is difficult because of the varying drugs and dosages represented, as well as the varying lengths of administration. The general opinion of the authors cited, however, is that thiouracil will decrease metabolic activity of anginal patients and reduce the frequency of anginal attacks. As can be seen by Table I, approximately ¾ of the patients developed a lowered metabolic rate; 18 of the 93 developed myxedema, a ratio of 1:5. It is interesting to note that in a series similar to the one here reported, 5 of 10 healthy males at Vermont State Hospital developed a lowered metabolic rate while taking thiouracil for 4 months, but none developed myxedema.

Material and methods. 6-n-propylthiouracil was procured as a powder,‡ weighed, and placed in 250 mg and 500 mg amounts in gelatin capsules. Each of the 8 patients received the following course of treatment:

250 mg	alt dei	× 8 days
250	daily	× 7
250	b.i.d.	× 7
250	t.i.d.	× 5
500	b.i.d.	× 6 wk
750	b.i.d.	× 6
1000	b.i.d.	× 6
1000	t.i.d.	× 6

The subjects were patients at the Arkansas State Hospital for Nervous Diseases at Little Rock, Arkansas. For a primary psychiatric diagnosis, 5 had schizophrenia, 2 had mental deficiency, and one had general paresis. Physically, no patient revealed hypothyroidism

TABLE I. Recapitulation of Literature on Effect of Thiouracil Derivatives on Normal Human Thyroids.

Reference	Disease	Total No. patients	BMR decreased	Developed myxedema	Drug*	Daily dose, mg	Duration, wk
Ben-ashur(1)	Angina	37	35	2	T	100–600	?
Dipalma, *et al.*(2)		8	2	2	T	(209 g, 250 days)	
Hollander, *et al.*(3)		10	1	1	Pt	50–200	12–33
Raab(4)		10	7	1	T	100–1200	4–26
Schoenwald(5)		3	3	3	Mt	100–600	?
Reveno(6)		8	6	2	T	200–600	6–33
					Pt	75–125	10–12
Waitzkin(7)		7	7	7	Pt	800–3000	8–40
Raab(8)	10 healthy patients at Vermont State Hosp.		5	0	T	400	16
		93	66	18			

* T = Thiouracil; Pt = Propyl-thiouracil; Mt = Methyl-thiouracil.

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‡ Generously supplied by the Lederle Laboratories.

prior to onset of treatment. In each instance, they had regular, ovulatory menstrual cycles as proven by weekly endometrial biopsies before therapy. Endometrial biopsies were obtained at weekly intervals before, during, and after propylthiouracil therapy. They were taken with a Randall suction curette and stained with hematoxylin and eosin. The letters "P" and "S" on Fig. 1 indicate "proliferative" and "secretory" endometrium, respectively. The character of the endometrium at the time of menstruation is depicted on this chart. A *proliferative* endometrium signifies failure of progesterone secretion, failure of corpus luteum formation, and thus failure of ovulation. A *secretory* endometrium, on the other hand, indicates that ovulation, corpus luteum formation and progesterone secretion have occurred. So "P" indicates an anovulatory cycle, and "S" indicates an ovulatory cycle. Biweekly red blood cell and white blood *cell counts* were made during the administration of the propylthiouracil to guard against idiosyncrasy to the drug. The *basal metabolic rate* was determined at weekly intervals before, during, and after completion of therapy.

Results. The data obtained from a single patient, D.K., are presented graphically in Fig. 1. Of the 96 menstrual cycles observed, only 3 were anovulatory, 2 of these occurring in Pt. D.K. (Fig. 1). All other cycles were regular and ovulatory, as indicated by the

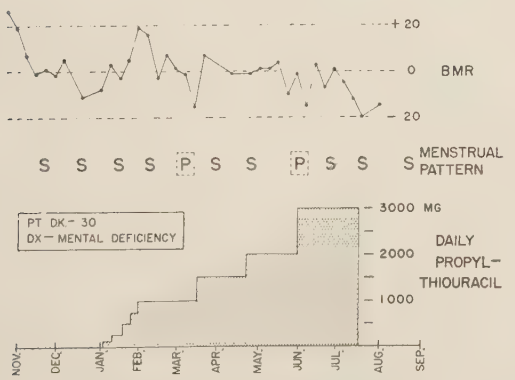

FIG. 1. Graphic illustration of the basal metabolic rate (BMR) and premenstrual endometrial pattern of an individual patient during the control period and while under therapy with the indicated amounts of propylthiouracil. For explanation of the letters "P" and "S," see text.

TABLE II. White Blood Counts during Propylthiouracil Therapy (43-51 Counts/Patient).

Patient	Highest	Lowest	Avg
C.	7500	4500	5450
H.	12400	5400	7800
L.H.	12200	4300	6850
L.	15800	4100	5150
B.	13800	5000	7650
S.	15300	7000	11400
K.	13800	4500	8200
W.	11000	4700	6700

endometrial biopsies. Correlating the propylthiouracil administration with the basal metabolic determinations, it may be seen that a plateau was reached before administration of the drug which was not significantly altered by the drug (Fig. 1).

A summary of the white blood cell counts is shown in Table II. In no case was a leucopenia found, although the biweekly determinations showed a rather wide variation. Fig. 1 shows minimal and maximal values, along with the mean value for each patient. The red blood cell determinations showed no changes of significance.

Discussion. In the majority of smaller experimental animals, guinea pigs, rats, mice, etc., hypothyroidism is easily induced in the normal animal by adding small amounts of thiouracil or its derivatives to the diet. According to the literature on man, about ¾ of euthyroid patients who receive therapeutic amounts of thiouracil develop clinical hypothyroidism and about one-fifth are said to develop myxedema.

With this in mind, 8 women were selected, because of the regular occurrence of ovulatory menstrual cycles as proven by weekly endometrial biopsies, and because of the absence of any signs of hypothyroidism, to be subjects to receive propylthiouracil in an attempt to induce hypothyroidism and precipitate menstrual abnormalities associated with the hypothyroid state. Propylthiouracil was administered in increasing doses from 250 mg to 3000 mg daily over a period of 7½ months, and was accompanied by no clinical evidence of hypothyroidism, no consistent alteration in the basal metabolic determinations, and no alteration in the menses of any of the 8 subjects. The occurrence of 3 anovulatory cycles

among the 56 occurring during therapy was interpreted as probably a normal occurrence unrelated to the drug. No toxic manifestations occurred in these patients, in spite of the high dosages of propylthiouracil utilized.

To verify that these psychiatric patients received the correct amounts of the propylthiouracil, it was dispensed by one of the authors personally. On 3 occasions, one of the patients vomited the material; this happened so infrequently, it was felt that it did not affect the final outcome of the observations.

None of the patients developed clinical manifestations of hypothyroidism. No palpable enlargement of the thyroid was noted. On the other hand, the attendants at the institution voluntarily offered the information that there was an increased activity and cooperation on the part of the study patients, which may be attributed to the increased attention they were receiving.

In view of the observations of Waitzkin(7), it is possible that the reason for the resistance to propylthiouracil noted in these patients lies partly in the fact that a 12-hour interval lapsed between doses. His patients reached the myxedema level when they received the drug at 6-hour intervals.

Summary. Propylthiouracil was administered to 8 euthyroid women over a period of 7½ months in large dosage. No evidence of hypothyroidism was noted, *i.e.*, no change of basal metabolic rate, no alteration of menstrual cycles, no clinical signs or symptoms of hypothyroidism, and no thyroid enlargement. No toxic manifestations were noted, even though during the final 6 weeks, each patient received 3000 mg of propylthiouracil daily in divided doses.

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Survival, Respiration and Morphology of Sarcoma-37 Cells Exposed to Liquid Nitrogen.* (19914)

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The survival of a variety of cells, tissues, and organisms exposed to subzero temperatures is well known. Reviews of some of the basic technics, results and theories pertaining to survival at low temperatures have been presented by Luyet and Gehenio(1), Luyet(2), Billingham and Medawar(3), and Parkes(4). In general it has been demonstrated that some of the deleterious effects of freezing can be avoided by ultra-rapid cooling and rewarming, by partial dehydration, and by pretreatment with hypertonic solutions of substances such as sucrose, glucose, ethylene glycol, and glycerol. The production of tumors *in vivo* from frozen tumor material has been known for almost 50 years, but the question of the actual survival of frozen tumor cells as opposed to the survival of a virus-like causative agent has been reemphasized(5). The survival of various types of tumor cells following freezing has been recently demonstrated by Walsh, Greiff, and Blumenthal(5), Ludwin (6), and Passey and Dmochowski(7). Walsh *et al.*(5) have observed a decrease in the growth potential of sarcoma-37 cells frozen at —30°C and —70°C, but not when frozen at —190°C and stored at —70°C.

It has been our aim to attempt a quantitative estimation of surviving cells by measuring

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the rate of oxygen consumption of thin slices of sarcoma-37 which were previously frozen under various conditions at —190°C. These results were verified by *in vivo* growth and histological examination of the tissues incubated *in vitro* after freezing. In preliminary experiments(8), a measurable respiration was demonstrated in slices of sarcoma-37 which were pretreated with 30% ethylene glycol, fast frozen in liquid nitrogen, rapidly thawed and then incubated for one hour in Krebs-Ringer-Phosphate solution. No respiration was detected in untreated frozen tumor slices. *In vivo* tumor growth occurred from both glycol treated and untreated frozen tumor material. The experiments reported here represent an extension of this work with improvements in technic which resulted in more accurate estimations as well as increased tumor cell survival.

Materials and methods. The physiological medium used throughout these experiments was a mixture of equal parts of Krebs-Ringer-Phosphate solution(9) containing 0.2% glucose, and partially neutralized horse serum. The serum was neutralized according to the procedure of Friend and Hastings(10). The sterile components of this serum medium were combined just before using since the mixture could not be stored in a refrigerator without the precipitation of phosphates. The pH of the medium was 7.4 at the beginning of the experiments and did not go below 6.9 after incubation. Sarcoma-37, originally procured from the National Cancer Institute, was maintained in Swiss mice by subcutaneous transplantation into the axillary region. Animals bearing 7- to 14-day-old tumors were killed by crushing the spinal cord at the base of the skull, and the tumor was then removed and placed in a small dish containing physiological medium. The obviously necrotic portions were trimmed off. The tumor was then sectioned with a Stadie-Riggs microtome(11) modified to produce slices 0.3 to 0.4 mm in thickness. Six to 10 slices weighing between 20 and 30 mg were selected according to uniformity of appearance and thickness. Usually one tumor yielded a sufficient number of slices for a single experiment. It was frequently impossible to obtain good uniform slices because the tumors normally contained numerous necrotic regions irregularly scattered throughout the mass. Each slice was carefully blotted with filter paper, moistened, but not saturated with physiological medium and then weighed with a micro-torsion balance to the nearest 0.1 mg. All respiratory data have been calculated on the basis of these moist weights. The slices were either returned to the physiological medium, or immersed for 2 minutes in 30% glycol solution. They were then floated onto thin pieces of mica, carefully spread flat and blotted. The mica preparation bearing 2 to 4 slices was immediately subjected to the low temperature. Under these conditions the treated slices were exposed to the action of 30% glycol at room temperature for 2½ to 3 minutes just prior to freezing. In our preliminary experiments(8) as well as in the experiments of Group I (Table I) in this series 30% ethylene glycol in water was employed. In all succeeding experiments 30% ethylene glycol in Krebs-Ringer-Phosphate solution was used.

The treatment hereafter referred to as *fast freeze* was accomplished by immersing the slices in liquid nitrogen for one to 2 minutes, then rapidly rewarming by immersion in 20 ml of physiological medium at room temperature. The warming bath served also as a rinse. The *slow freeze* treatment was accomplished by placing the preparations in the cold nitrogen gas just below the rim of a 500 ml Dewar flask half filled with liquid nitrogen. After freezing was apparent (about 30 sec.) the tissues were gradually lowered through the cold nitrogen gas which had a temperature gradient of approximately —40° to —190°C. They were then immersed in the liquid nitrogen for one to 2 minutes. These tissues were thawed in air at room temperature. When the tumor slices were slowly frozen more than once the succeeding freeze was started immediately after the tissue appeared to thaw. These procedures for fast and slow freezing were essentially similar to those employed by Luyet(2). A more constant and reproducible method of slow freezing was accomplished by the treatment referred to as *very slow freeze*. In this procedure the tumor slice preparations were suspended in air in a deep freeze box at

TABLE I. Qo_2* of S-37 Slices Frozen in Liquid Nitrogen With and Without Pretreatment in Ethylene Glycol Solution.

Group	Exp. No.	Incubation time, hr	Qo_2 controls: Not treated	Qo_2 controls: 30% glycol	Slow freeze: Not treated Qo_2	Slow freeze: Not treated % C†	Slow freeze: 30% glycol Qo_2	Slow freeze: 30% glycol % C	Fast freeze: Not treated Qo_2	Fast freeze: Not treated % C	Fast freeze: 30% glycol Qo_2	Fast freeze: 30% glycol % C
I		Slow freeze 2 times. Glycol in water. No rinse										
	1	3	1.20		.08	7					.35	29
	2	3	.58	.56	.01	2					.08	14
	3	2	.72	.74	.10	14					.21	28
	4	2	.75		.03	4					.18	24
	5	2	.63								.15	24
	6	4		.51							.11	22
	Mean		.77		.06	7					.18	24
II		Slow freeze 2 times. Glycol in Krebs-Ringer-Phosphate										
	7	2		.67			.33	50			.37	55
	8	2		.51			.61	120			.49	96
	9	4		.48					.06	13	.20	42
	10	4		.55							.27	49
	11	4		.39			.13	33			.30	77
	Mean			.52			.36	68			.33	64
		One slow freeze										
	12	4		.55			.28	51			.34	62
III		One very slow freeze. 2 rinses in all. Glycol in Krebs-Ringer-Phosphate										
	13	4		.65			.31	48			.41	63
	14	4	.96	.83	.00	0	.54	65				
	15	4	.90	1.04	.03	3	.58	36				
	16	4	1.03				.39	38			.41	40
	17	4	.50		.00	0			.00	0		
	18	4	.94		.00	0			.02	2		
	19	4	.78	.70					.00	0	.32	46
	Mean		.85	.81	.01	1	.41	47	.01	1	.38	50

* Qo_2 equals the quantity of oxygen (NTP) consumed/mg moist tissue/hr during the final 1 to 2 hr incubation. Tissues were incubated in 1 part neutral serum to 1 part Krebs-Ringer-Phosphate at 37°C in an atmosphere of oxygen.

† Refers to % of control value.

—25°C. This temperature is about 10°C lower than the initial freezing point of a 30% glycol solution. The slices appeared well frozen within 5 minutes after which the temperature of the box was gradually lowered with dry ice over a period of 10 to 15 minutes to approximately —40°C. From this point on the slices were slowly cooled to the temperature of liquid nitrogen and thawed in the same manner as described for the *slow freeze*. After *thawing* the slices were rinsed, except as noted in Table I, then blotted and placed in 5 ml micro-Warburg vessels containing 1.2 or 1.3 ml of the neutral serum medium. The control slices were allowed to remain in the incubation medium in small dishes at room temperature while the experimental slices were being prepared. The oxygen uptake was determined manometrically by the direct method of Warburg(10) during incubation periods ranging from 2 to 5 hours at 37°C in an atmosphere of pure oxygen. Control slices were always prepared and measured in duplicate, while the experimental slices were done in duplicate, triplicate or quadruplicate. All tumor slices were fixed in Bouin's solution at the end of the experiments. Representative sections, cut at right angles to the plane of the slice, were stained with hematoxylin and eosin for microscopic examination.

As supplementary evidence of growth and survival 2 complete experiments were conducted in which groups of 4 sarcoma-37 slices were given the fast and slow freeze treatments with and without glycol. The slices were then incubated with controls under sterile conditions in the Dubnoff metabolic shaking incubator(12,13) for 18 hours at 35°C in an

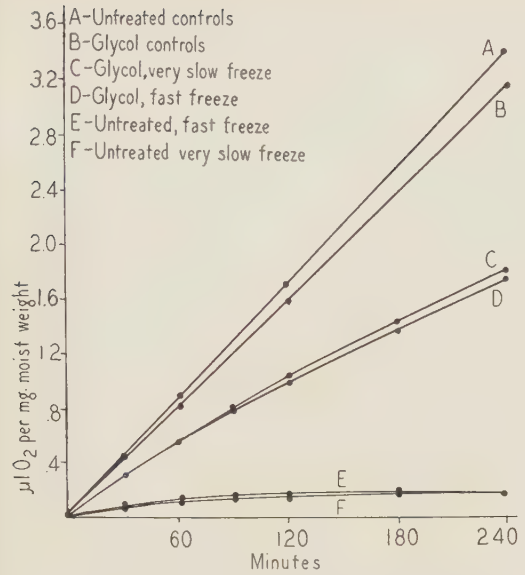

FIG. 1. Oxygen uptake of slices of sarcoma-37 previously frozen in liquid nitrogen with and without pretreatment in 30% ethylene glycol in Krebs-Ringer-Phosphate solution. Slices were incubated at 37°C in equal parts of partially neutralized horse serum and Krebs-Ringer-Phosphate solution.

atmosphere of 5% CO_2 in O_2. The incubation medium was a mixture of equal parts of normal horse serum and Krebs-Ringer-Phosphate solution. At the end of the experiments half the slices were reinjected into mice and the rest prepared for histological analysis.

Results and discussion. The essential respiratory data are presented in Table I. Each Qo_2 value represents the mean of the duplicates, etc., and was calculated on the moist weight basis. The oxygen consumption curves in Fig. 1 were constructed from the means of all the individual determinations in the experiments of Group III, which is the most uniform and complete group. The general characteristics and relative position of these curves is representative of all the experiments in Table I. All the controls respired at a constant rate throughout the incubation period. The respiration rate of the glycol treated frozen slices usually decreased continuously throughout the first 60 to 120 minutes of incubation and thereafter respired at a constant rate which was approximately 50% of the control values. The untreated frozen slices usually but not always respired at a significant level during the initial period and then after 60 to 120 minutes incubation consumed no measurable quantities of oxygen. The experimental Qo_2 values listed in Table I are those obtained during the final period of constant oxygen consumption.

The neutral serum incubation medium gave results which were superior to those obtained in the Krebs-Ringer medium used in the earlier experiments(8). Histological examination of fresh sarcoma-37 slices incubated for 60 minutes in Krebs-Ringer solution revealed a morphological deterioration of the cells which was not evident even after prolonged incubation in the neutral serum medium.

The drop in the pH of the medium during the experiments was correlated with the rate of respiration. In the controls the pH dropped from 7.4 to 6.9 or 7.0, the glycol treated frozen tissues dropped to 7.0 or 7.1, while there was practically no change in the untreated frozen tissues. This is consistent with the ability of tumor cells to produce acid under aerobic conditions.

The variations in the Qo_2 of the controls are explainable on the basis of the relatively large variations in the amount of necrotic material present. In general the microscopic appearance of the control slices was found to be quite representative of the experimental slices. There was one notable exception in Exp. 8 (Table I) in which the control slices contained much more necrosis than the experimental slices. A comparison between the morphological and respiratory data indicated that sarcoma-37 tissue without any necrotic regions would yield a Qo_2 (moist weight) of about 1.4 to 1.5.

A comparison of the treated and untreated controls in experiments 2, 3, 14, 15, and 19 demonstrates no effect of the glycol treatment on oxygen consumption. The superiority of 30% glycol in Krebs-Ringer solution over glycol in water is indicated by comparing the results of the fast frozen glycol treated tissues of Group I with those of Groups II and III.

The results obtained from the microscopic examination of the fixed and stained slices confirmed and extended the respiratory data. No constant significant differences were noted between the untreated and glycol treated controls after 4 hours incubation. The cells were

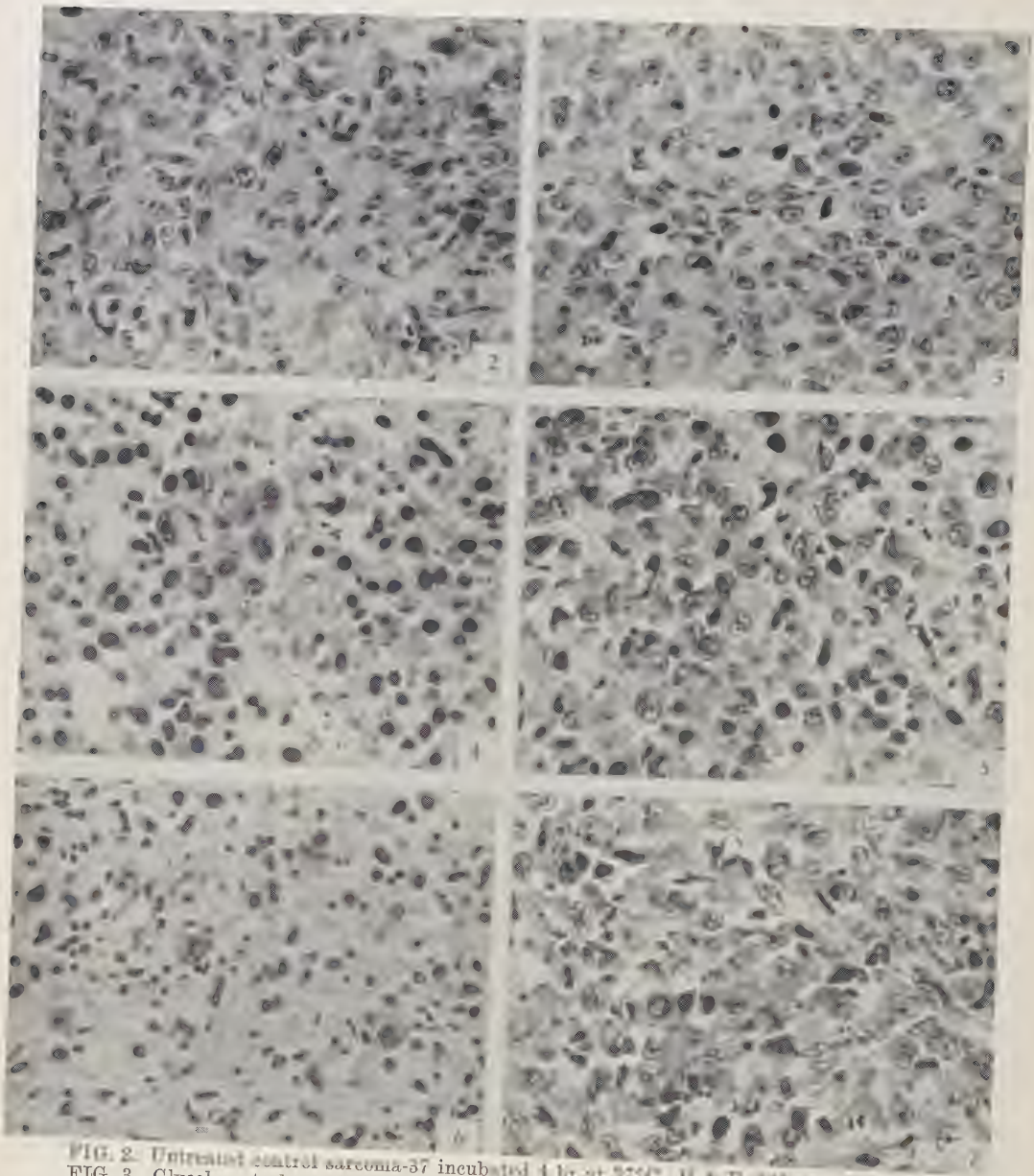

FIG. 2. Untreated control sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 3. Glycol control sarcoma-37 treated 3 min. in 30% ethylene glycol in Krebs-Ringer-Phosphate, then incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 4. Untreated, fast frozen sarcoma 37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 5. Glycol pretreated, fast frozen sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 6. Untreated, very slowly frozen sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 7. Glycol pretreated, very slowly frozen sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.

normal in appearance with frequent mitotic figures (Fig. 2 and 3).

Histological comparisons between the 2- and 4-hour incubated frozen tissues indicated that the shorter period was insufficient to give a clear morphological distinction between the surviving and the killed or irreversibly injured cells. The microscopical analyses are conse-

quently based primarily on the 4-hour incubated slices. There was no apparent difference between the glycol treated fast and slowly frozen slices, including the multiple freezings in Group II. The majority of the surviving cells of the glycol treated frozen tissues (Fig. 5 and 7) were somewhat inferior to the controls. There was a slight cytoplasmic eosinophilia and hyperchromatic appearance of the nuclei. Mitotic figures were quite abundant. Many of the surviving cells appeared essentially normal, but many others showed signs of severe injury. About half of the cells of these slices were estimated to have been killed or irreversibly injured by the low temperature. Such cells after 3 to 4 hours incubation were reduced in size, possessed a marked cytoplasmic eosinophilia, and had a small pyknotic nucleus which was frequently disintegrating. Degenerating mitotic figures were frequently recognized. Also evident were completely necrotic areas identical in appearance with the necrotic areas of the control incubated slices.

The microscopic appearance of the fast and slowly frozen untreated slices was also essentially the same (Fig. 4 and 6). They differed significantly from the frozen glycol treated tissues since very few of the cells were estimated to be surviving. The surviving cells possessed a pale to slightly eosinophilic cytoplasm and a hyperchromatic nucleus which was frequently more lobulated and vesicular than normal. Occasionally some of the cells were nearly normal and a few mitoses were observed. The completely necrotic and degenerating regions of these preparations were similar to the glycol treated frozen slices. The only significant respiration of untreated frozen slices occurred in Exp. 1, 3, and 9. In these 3 experiments 5 to 15% of the cells were judged to be surviving while in all other experiments less than 1% were considered viable. It appears that a reliable measure of respiration was obtained during the 4-hour incubation period only if approximately 5% or more of the cells were surviving. Examination of the frozen slices which were incubated for 18 hours verified the above results. Most of the surviving cells in the glycol treated frozen preparations were essentially normal in appearance and mitotic figures were numerous. The untreated cells which survived freezing were on the average still morphologically inferior to the glycol treated frozen cells, but many were approaching a normal appearance and mitotic figures were fairly numerous. Tumors developed *in vivo* from both glycol treated and untreated frozen cells incubated for 18 hours. However, those tumors which developed from the untreated fast and slowly frozen tissue were retarded in growth rate as compared with the others. This latter fact requires additional investigation and may be significant in connection with the results of Walsh *et al.*(5) previously mentioned.

The results of these experiments seem to indicate that we were dealing with the problem of estimating the survival of cells which were reversibly injured to a greater or lesser extent by exposure to very low temperature. The Krebs-Ringer-Phosphate solution used in the first experiments(8) as an incubation medium was inadequate. The serum medium used in the present experiments filled the requirements since it sustained survival and growth of normal and injured sarcoma-37 cells for prolonged periods.

The decrease in the rate of respiration of the frozen slices during the first one to 2 hours of incubation may be explained by a diffusion of the enzymes, cofactors and substrates from the dead and dying cells into the surrounding medium. The dilution of the components of these enzyme systems would result in a marked decrease, if not an almost complete cessation of their activity. DeRobertis and Nowinski(14) measured the respiration of slices of guinea pig liver which was previously frozen en masse in liquid air. They obtained respiration during a 30-minute incubation period which was 28.5% of the control slices. This decreased respiration was interpreted as being due in part to the diffusion of substrates out of the degenerating cells(15). The unwashed, untreated and twice slowly frozen slices of Group I, Table I, are somewhat comparable to the above experiments. The respiration of these slices during the first hour of incubation was 26% of the controls before falling off to nearly zero. The un-

treated frozen tumor slices in the third group of experiments (Table I and Fig. 1) were rinsed twice after freezing before incubation and the respiration of these tissues was only about 14% of the control during the first hour. It thus appears that the respiratory activity not associated with surviving cells was decreased by rinsing and was not detectable in these experiments after one to 2 hours of incubation.

The constant rate of oxygen consumption during the last 2 hours of incubation is considered to be due solely to the activity of surviving cells and as such to be a quantitative measure of survival. Though the accuracy of the method may be 5%, the error in these experiments could be larger due to the variable nature of the tumor material. On the basis of the respiratory data it is therefore estimated that approximately 50 to 60% of the sarcoma-37 cells pretreated with 30% ethylene glycol in Krebs-Ringer solution survived both fast and slow freezing in liquid air. No additional destructive effect of 2 successive slow freezes was observed. These results on glycol treated material are supported by the cytological analyses. It seems quite probable that if the conditions of exposure to glycol could be made uniform for all cells of the slice, 100% survival could be obtained.

The good survival of glycol treated, frozen sarcoma-37 cells is in agreement with the work of others using glycol, glycerol, sucrose, glucose, etc., in low temperature experiments with a variety of living material(1-4,7). The nature of the protective action of glycol against the deleterious effects of low temperature is obscure and requires further work. It could be due to the removal of some of the free water of the cell by reason of its hypertonicity, or to the penetration of the glycol into the cells and thus either replace some of the water or bind it so it is no longer free to crystallize. The penetration of the glycol into the cells seems to have some support from the observation that fresh tumor slices immersed for 2 minutes in 30% glycol solution appeared more transparent and turgid than fresh untreated slices. A similar observation has been recorded for surviving skin grafts in glycerol(3).

The surviving cells in the untreated frozen preparations were so few that their respiration was not usually detected. Proof of survival in these experiments rests primarily with the microscopic analyses of 4- and 18-hour incubated slices and the *in vivo* development of tumors.

No effect of the rate of cooling was observed with either ethylene glycol treated or untreated tumor tissue in these experiments. Numerous workers have reported apparently contradictory results regarding the relative injurious effects of fast versus slow rates of freezing(1-5,16). Most recently, Billingham and Medawar(3) working with rabbit epidermis, have stated that slow freezing is preferable to quick freezing on every ground for comparison. Since our slowly frozen preparations were thawed slowly whereas the slowly frozen preparations of Billingham and Medawar were thawed rapidly, direct comparisons are not valid. These authors as well as others(1,2) have shown that the rate of thawing is significant. The original purpose for slow freezing and thawing, and multiple freezings of the glycol treated cells was to attempt to kill all the cells by freezing. This was not accomplished. It was also expected on the basis of Luyet's works(1,2) that a better survival might be obtained in the untreated rapidly frozen and rapidly thawed preparations than in the slowly frozen and slowly thawed preparations. The fact that we did not obtain better survival in our untreated rapidly frozen preparations might be due to a slower rate of cooling than employed by Luyet for vitrification of cell contents. Our preparations were 3 to 4 times thicker than recommended and were cooled by direct immersion in liquid air rather than in a less volatile cold bath.

Occasionally some extraneous tissues included in the tumor slices were judged to survive freezing when pretreated with glycol. Thus the cellular elements of fibrous connective tissues, fat cells and skeletal muscle fibers occasionally retained their normal morphological appearance after freezing and incubation.

Summary. 1. The survival of mouse sarcoma-37 cells frozen in liquid air has been

quantitatively estimated by determinations of the rate of cellular respiration. The results were checked by morphological examination of the frozen incubated tissue and by *in vivo* growth. 2. Measurements of the rate of respiration were reliable as an index of survival if the cells were incubated for more than 2 hours at 37°C following freezing, and if they were suspended in a physiological medium compatible with normal survival and recovery of injured cells. 3. Krebs-Ringer-Phosphate medium was unsuitable, but equal parts of partially neutralized horse serum and Krebs-Ringer-Phosphate provided an adequate medium. 4. The respiration and survival of sarcoma-37 cells, pretreated for 2½ to 3 minutes in the ethylene glycol in Krebs-Ringer solution then frozen in liquid air was 50 to 60% of the unfrozen controls. 5. The respiration of untreated frozen sarcoma-37 was not usually significant. Morphological analyses of these tissues indicated fewer than 1% surviving cells in most experiments. 6. No significant difference in survival was observed between the rapidly and slowly frozen tissues. 7. Good correlation was observed between the morphological and respiratory characteristics of the frozen incubated tumor cells.

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Role of Heparin in *in vitro* Production of Alpha$_1$ Lipoproteins in Human Plasma. (19915)

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Recent studies on lipid transport and metabolism(1,2) have demonstrated a marked alteration of several classes of low-density lipoproteins in plasma following heparin injection. Further information concerning the nature and mechanism of action of this "lipemia clearing system" has recently been reported from this laboratory(3,4). In these latter studies it was found that the lipoprotein changes previously observed *in vivo* could also be demonstrated in an *in vitro* system employing several purified plasma proteins.

The present communication deals with the nature of the products formed during the clearing reaction. The data below demonstrate that alpha lipoproteins are formed *in vitro* in the plasma of individuals containing the essential components of the clearing system.

Methods. Eight healthy young adult males

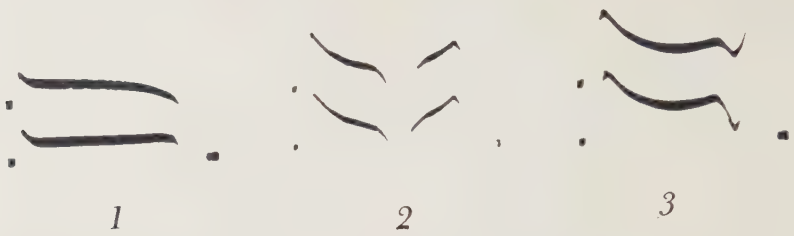

FIG. 1. Lipoprotein boundaries before and after *in vitro* clearing of human plasma. Upper image: immediately after intrav. heparin. Lower image: after 3 hr incubation. The lipoproteins are ascending at a saline density of 1.21. Frame 1: 50000 r.p.m. Frame 2: 12 min. at max speed. Frame 3: 30 min. at max speed.

were treated in the following manner. Approximately 3½ hours following a high-fat breakfast, 25 mg of heparin (2.5 ml of Liquaemin) were injected intravenously. Within the next two minutes, 80-120 ml of blood were withdrawn and immediately transferred to a stainless-steel coil immersed in ice water. This rapid cooling delayed the enzymatic clearing reaction. Either heparin or Sequesterene was used as anticoagulant. The choice of anticoagulant had no effect on the results described below. Plasma was separated at 4°C by centrifugation for 10 minutes at 4,000 rpm. The clearing activity of 2 aliquots of plasma was irreversibly destroyed by bringing them to saline densities of 1.063 and 1.21 respectively by the addition of appropriate quantities of a concentrated NaCl-KBr solution. The remainder of the plasma was incubated at 37°C for 3 hours to allow the clearing reaction to continue. Two additional aliquots were then adjusted to densities of 1.063 and 1.21 respectively. All 4 samples were centrifuged in the Spinco preparative centrifuge at 40000 rpm (105,400 x Gravity) for 18 hours at 4°C. The plastic tubes were sliced by means of a sharp blade in a rigid holder at identical levels for each pair. The top samples at a density of 1.21, containing both low-density and alpha lipoproteins, were centrifuged simultaneously in the wedge and ordinary analytical cells of the Model E Spinco ultracentrifuge. These runs were made at 25 ± 1° at 59780 rpm. Photographs were taken during acceleration at 10000 rpm, 30000 rpm, and 50000 rpm, and at 0, 2, 6, 12, 20, and 30 minutes after attaining maximum speed.

In the plasma samples at a density of 1.063, the low-density lipoproteins accumulated at the top of the centrifuge tube. The alpha-lipoproteins, having a density greater than 1.063, sedimented toward the bottom of the tube. The 10 cm long tubes were therefore guillotined 19 mm from the top, where the solution was lipoprotein-free. The top and bottom material was quantitatively recovered and each sample analyzed in duplicate for lipid content. Conventional methods of extraction were tried, but the following proved the most satisfactory. Proteins were precipitated with sodium tungstate and the water soluble substances were removed by washing. The lipids were then extracted from the proteins with aqueous ethanol-ether. Aliquots of the extract were analyzed for free and total cholesterol by the method of Schoenheimer and Sperry(5); for lipid phosphorus by the method of Fiske and SubbaRow(6); and for total lipids by a recently described colorimetric method(7). Lipid analyses were carried out on six of the eight cases reported here.

Results. During the 3-hour incubation of all plasma samples examined, analytical ultracentrifugation revealed the qualitative shifts within the low-density lipoprotein spectrum from those of lower to those of higher density, previously described by Graham *et al.*(2) in their *in vivo* studies on the effects of intravenous heparin administration. In addition, however, there was a significant quantitative increase in the alpha$_1$ lipoprotein component, a change not previously reported. Fig. 1 illustrates these changes in case No. 4 (Table I). It may be seen in frame No. 1 that the S_f 51-300 class molecules (S_f 10-80 at density 1.063) have disappeared in the 3-hour sample and, in frame No. 3, that there is a quantitative increase in the alpha$_1$ lipoproteins. The

TABLE I. Lipoprotein and Lipid Shifts Occurring *in Vitro* in Human Plasma, 8 Cases, During a 3-Hr Incubation Period Following Intravenous Heparin. Quantities are presented as mg/100 ml of plasma. The lipoprotein changes were quantitated in the analytical ultracentrifuge. The chemical changes represent lipid shifts from the top to the bottom of preparative tubes containing plasma at a saline density of 1.063.

S_f 100-300* S_f 27-80 †	S_f 74-100 S_f 20-27	S_f 51-74 S_f 10-20	S_f 31-44 S_f 3-8	S_f 2-8 —	Phospholipid	Ester cholesterol	Neutral fat and fatty acid
0	0	—19	+ 9	+ 3	5	1	20
—17	— 6	—45	—15	+17	12	3	38
0	—15	—37	—14	+35	—	—	—
— 5	—13	—28	+10	+43	17	—	75
0	—15	—15	0	+44	12	2	55
0	—	—17	—	+51	19	3	62
—28	—34	—31	—43	+61	—	—	—
—54	— 5	— 2	+18	+75	28	5	100

* Flotation rate at density 1.21.
† " " " " 1.063(8).

quantitative results for 8 cases studied are presented in Table I. In several samples, the alpha$_1$ lipoproteins migrated upward as two distinct components, which we have termed alpha$_{1,2}$ and alpha$_{1,1}$ lipoproteins respectively. The more slowly rising component, alpha$_{1,1}$, appears to increase specifically during the action of the clearing system. These changes have never been observed in control samples obtained before heparin injection.

Quantitation of the very low density, high molecular weight lipoproteins of S_f 300 and above cannot be satisfactorily carried out in the analytical ultracentrifuge since these molecular classes are not sufficiently discrete to yield boundary resolution, and, in addition, scatter light intensely. Lipid analyses, however, of the samples at a density of 1.063 indicated that, during the clearing process, lipid disappeared from the top of the tube and could be quantitatively recovered from the bottom. Triglycerides and fatty acids, phospholipids, and, to a lesser extent, cholesterol esters diminished in the top fraction and increased in the bottom. The extent of these lipid shifts is presented in Table I. It should not be inferred that triglycerides were incorporated into the alpha$_1$ lipoprotein molecules. Certain unpublished chemical data indicate that, during the clearing process, triglycerides in the low-density lipoproteins are hydrolyzed. In the present experiments, therefore, the fatty acids and resulting soaps would have distributed themselves equally throughout the sample, thereby raising values in the bottom fraction. At the present state of our investigations, the figures in Table I are significant only to the extent of indicating the amounts of lipid liberated from the low-density lipoproteins.

Summary. 1. An absolute increase in high-density alpha$_1$ lipoproteins, and a concomitant decrease in certain low density lipoproteins, have been demonstrated by ultracentrifugal and chemical means. 2. These changes occurred *in vitro* in plasma obtained after heparin injections and suggest, on the basis of earlier studies on the nature of the lipemia-clearing reaction, an enzymatic conversion of one lipoprotein class to another.

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Quantitation of Biological and Other Data by Photoelectric Measurement of Areas.* (19916)

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The measurement of areas, regular or irregular, planar or projected, is frequently necessary in engineering and physical sciences. More recently, the method of area measurement has found increasing application in the computation of data obtained in various types of biological investigations and laboratory work. The usual method of area measurement calls for the laborious and tedious use of the polar planimeter. To avoid the disadvantages involved, it seemed desirable to develop an apparatus which would be suitable for the rapid and accurate determination of a wide range of areas (including very small ones), such as may be encountered in various laboratory technics in current use. A versatile instrument, utilizing the photoelectric measurement of intercepted light, and easily and inexpensively constructed in the laboratory from readily available components, has been devised. In simplicity, rapidity, and accuracy, its use is especially advantageous for the computation of the areas of electrophoretic patterns.

Description of apparatus. In order of arrangement, the essential components consist of a uniform light source, a condensing lens, a base plate surface at which the sample intercepts the light, another condensing lens, and a photoelectric cell connected to a galvanometer and a variable resistance.

The arrangement and design of the photometric apparatus are shown in Fig. 1. The light source consists of a 100-watt photo-enlarger-type (No. 212) lamp bulb in the Condensor Head (A) of a Kodak Precision Enlarger. The focusing (objective) lens, bellows, and the lower half of the condensing lens system, normally parts of this unit, are removed, leaving in place only the upper condensing lens (U). A 60 cycle, 120 watt voltage stabilizer of input 95-130 volts, output 115 volts (Raytheon Mfg. Co.) is required for the maintenance of constant voltage to the light source. The column holding the modified enlarger head is detached from its base and bolted to the leveled top of a light-tight, cabinet-type table base (B). In this top an aperture (O) 7¼ x 7¼ in. is cut. A recessed shoulder (S) ⅛ in. deep and ⅜ in. wide is machined along the perimeter of the aperture and serves to receive the various types of sample base plates (P) *e.g,* 8 x 8 in., which may be required. Usually, double thickness clear window glass may be used, or, for some applications ¼ in. metal sheet (Dural), the edges of which are machined to fit the aperture with upper surface flush with the table top. The metal base plate serves to hold area specimens on thin glass plates (G) (as in some applications, 9 x 12 or 8 x 10 cm). An aperture is likewise cut in the center of the metal plate, to permit rapid and reproducible placement of these glass specimen plates on a squared shoulder, recessed so that the glass plate is flush with the top surface of the holder. A finger indentation or tab of tape serves for the withdrawal of base plates and glass specimen holders.

The light source and base plates are centered and aligned. Sources of stray light are eliminated and possible reflecting surfaces are coated with Kodak Brushing Lacquer No. 4, dull black. *Flexaframe* (Fisher) assembly units (F), consisting of ½ in. rods, connectors, and feet, form the structural support for the focusing lens (L) and photoelectric cell (C) located below the table top. The vertical rods are aligned in proper position and fastened to top and floor of the cabinet by means of the foot units. The lens, 6 in. in diameter and 1½ in. thick at the center, of focal length approximately 10 in., is mounted in a properly leveled holder, in a centered position directly below the table top. With the light source in posi-

* The opinions expressed are the authors' and do not necessarily reflect those of the Navy Department.

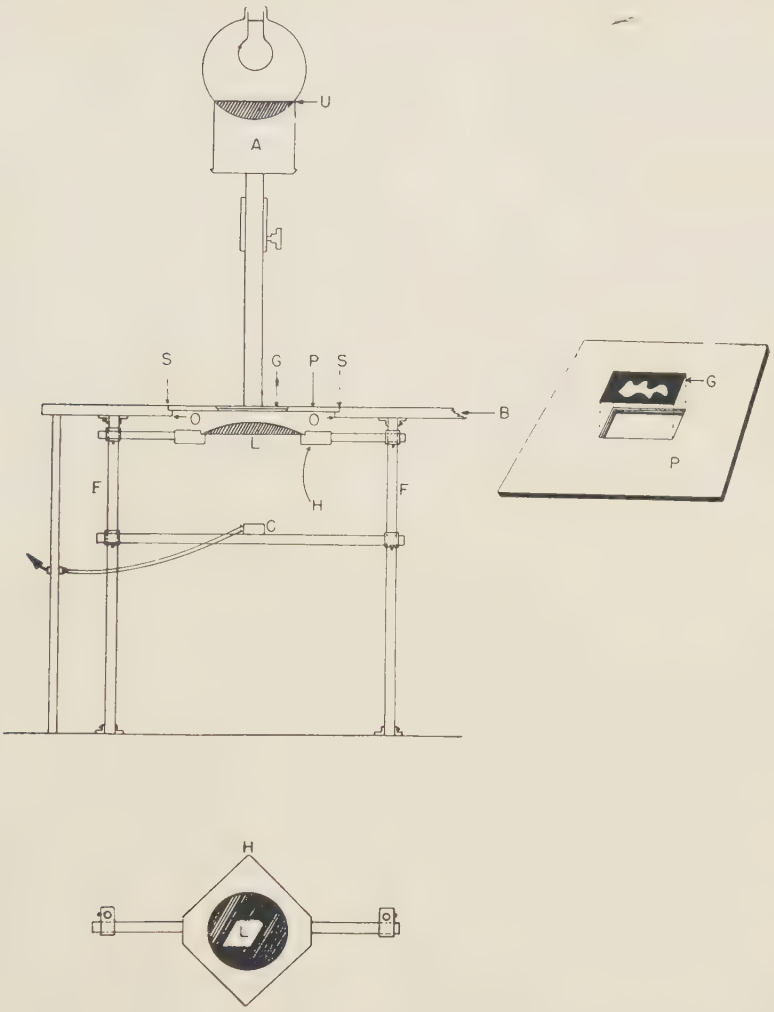

FIG. 1. Construction of areameter as described in text.

tion (usually at the highest level) about 37 in. from the table top, the photocell (Weston Photronic No. 594 RR or 856 RR) is fixed at a level (approximately 12 in. below the lens) such that the rectangular image of the condenser head aperture appears on the active surface of the cell in the maximum size circumscribed by the bakelite case.

The *photo current* generated by the cell is measured by a Rubicon galvanometer (Cat. No. 4625), sensitivity 0.006 μA/mm, resistance 1160 ohms, period 2.4 seconds, critical damping resistance, 7,800 ohms. The top of the galvanometer box is surmounted by an aluminum sheet metal cover with slides and back extended downward to shield against the effect of air currents, with a protecting piece of glass in front of the scale, for the same purpose. A rack and pinion device cemented to the front of the glass scale and operated manually by a knurled knob through the top of the box, permits fine control of scale movement to right or left.

A 180 ohm Helipot (Beckman Technical Laboratories) variable resistance, in parallel with the galvanometer, allows light current adjustments to a wide range of light intensities. A single pole double throw switch of high quality, is placed in the photocell circuit. In the neutral position, it serves to avoid wide swings of the galvanometer off the scale, whenever, as between readings, or when adjust-

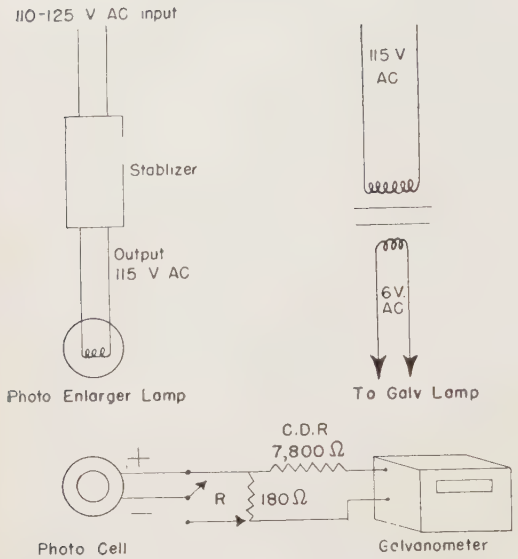

FIG. 2. Electrical circuits of areameter.

ments are being made for them, the cell is exposed to light of high intensity. The electrical circuit is shown in Fig. 2.

Procedure. The apparatus should be used under conditions such that environmental light does not, to any perceptible extent, affect the response of the photocell. To this end, there may be required a somewhat darkened room, or the use of a photographer's black cloth as a light shield between the enlarger lamp and the table top. The enlarger lamp is turned on, and usually allowed to attain a steady state over a period of 10 minutes. During this time, adjustment for dark current is made by covering the table top aperture with a sheet or flat piece of opaque material, closing the photocell switch to the galvanometer circuit position, and adjusting the galvanometer reading manually, exactly to zero.† A standard, known, transparent area masked by an opaque field, on a base plate corresponding exactly to that used for the specimen areas to be measured, is inserted in place and the galvanometer reading is adjusted to 100 electrically, by means of the variable resistance R (Fig. 2). The unknown specimen area is then immediately put in position, and the galvanometer reading observed. The full light current adjustment (the 100 setting) should be checked at frequent intervals between measurements of unknowns; the dark current zero should also be noted, although less often. To facilitate readings to 0.1 scale division a hand magnifier is used. Operational details and technics for various types of area measurements are best provided by the examples of experimental results given in the following.

Calibration and calculations. For the instrument described, the measured area must fall within the circumference, and hence be less than that (126.72 cm^2 or 19.64 $in.^2$) of a circle 5 inches in diameter. Measurements may be made of opaque (or less frequently, translucent) areas on a transparent background, or preferably, of transparent areas on a dark background. In effect, all measurements, whether of clear or opaque areas, are made by comparison with a *known,* transparent standard area set at a galvanometer reading of 100. Thus, the unknown area to be measured will be calculated as $Ta = R \cdot A/100 = Rm$, or $Oa = (100—R)\ A/100 = (100—R)m$ where Ta, Oa, and A denote unknown transparent, opaque, and known standard transparent areas, respectively, R is the reading of the unknown area, and m is the slope constant for all readings made against the known area with its galvanometer reading set at 100.‡ *Permanent primary standards* for calibrating the areameter over a range of

† It is good practice at all times when the instrument is not in use, to keep the switch in the position which short circuits the photocell. The photocell switch is closed to the galvanometer circuit only when galvanometer settings or readings are made.

‡ It is not necessary to have the transparent background area either known or calculable if measurements are made against a known, standard, opaque area provided the transparent background is kept constant and the same for any given set of measurements. The calculations for unknown transparent or opaque areas will then be made, respectively, according to the following equations:

$$Tu = \frac{R_{Tu} \cdot O_k}{S - R_{Ok}} \quad \text{or} \quad O_u = \frac{(S - R_{Ou})\ O_k}{S - R_{Ok}}$$

Where T and O are transparent and opaque areas, k and u are known and unknown, R is the appropriate reading, and S is the galvanometer setting for the transparent background, held constant for any group of unknown readings against a given opaque standard.

areas, and for testing the linearity of the instrument are made from 1/64 in. ground steel stock (Brown and Sharpe Mfg. Co.). A series of rectangular areas ranging from 0.402 to 38.712 cm^2 (0.0625 to 6.00 $in.^2$) ± 0.005 cm (± 0.002 in.) in each dimension are machined from the same pieces of stock. These metal standards are then used directly as opaque areas, or as templates for tracing and cutting secondary standards on taped glass plates as described in the following. In the latter case, the area or its background is peeled from the glass to leave either a transparent or opaque standard silhouette of identical size and shape as the metal template.

Experimental results. Clean glass base plates§ 8 x 8 in. were covered on one side with black Scotch brand tape No. 22, 5 in. wide (Minnesota Mining and Mfg. Co.). Well centered on individual plates, circles from 12.70 to 2.54 cm (5 to 1 in.) in diameter, were cut with a sharp, pointed graver (X-Acto) and peeled off the glass surface of the plates, to yield a series of transparent circular areas. The 12.70 cm circle was set in place, covered with a piece of cardboard, and the galvanometer set at zero. The cardboard cover was removed and the galvanometer reading adjusted to 100. In succession, the other plates were substituted for the 12.70 cm circle and the readings noted.‖ Table I indicates that the results are in accordance with the theoretical requirement that when the illumination is uniform, the intensity of light transmitted through the base plate be proportional to the cross sectional area of the transmitted light beam. The observed galvanometer reading values plotted against areas lie on or close to the theoretical line passing through the origin.

TABLE I. Proportionality of Light Intensity to Area of Transparent Circles of Known Diameters.

Diam. of circle, cm	Galvanometer scale reading*	Calc. area, cm^2	Observed area, cm^2
12.70	100	126.72	—
10.16	64.3, 64	81.10	81.30
7.62	36.2, 35.9	45.62	45.68
2.54 + 5.08	19.8, 19.6	25.36	24.97
5.08	15.9, 15.7	20.26	20
2.54	3.9, 3.9	5.10	4.97

* Results of 2 experiments performed on different days.

In another test, the accuracy of area measurements and the reproducibility of fabrication of the standard metal templates were determined. A succession of individual 2.540 cm metal squares was placed on a taped base plate within a transparent circular area of 124.58 cm^2 (19.39 $in.^2$), the galvanometer reading of which was initially set at 100. Table II indicates these results, which could be obtained repeatedly, regardless of the placement of the templates within the clear area, whether centered, randomly scattered, or distributed fanwise along the periphery of the circle.

To test the versatility of the instrument especially in respect to the accuracy of estimation of smaller areas, measurements were made with various base plate standard areas set at 100 (Table III). As would be expected, the maximum accuracy for any area measurement was obtained by the use of a standard area approaching in size that of the unknown. Thus, a 6.425 cm^2 (1 $in.^2$) area was measured more accurately against a 3 $in.^2$ standard set at 100, than against a 122 cm^2 (19 $in.^2$) standard (Table II). Opaque squares 1.606 cm^2 (0.25 $in.^2$) and 0.402 cm^2 (0.0625 $in.^2$) were measured to within one per cent of true value against a transparent 6.425 cm^2 area on tape background, set at 100. Indeed, the limitation as to the smallest areas accurately measurable, aside from the operator's ability to approximate fractional scale division readings, lies only in the accuracy with which a suitable small standard reference area can be made, and in the intensity of light available for measurement. Thus, by lowering the light

§ The most satisfactory glass for this purpose was that obtained by immersing exposed and otherwise useless photographic plates in hot soapy water to remove the emulsion.

‖ These measurements could be made by superimposing the glass plate of each of the smaller areas on top of the standard 12.700 cm circle area. In that case, the galvanometer reading would be set at 100 for the 12.700 cm circle area plate covered by a clear glass plate. Obviously, all of the glass used in any one series of measurements must be uniformly the same in quality and thickness. Transparent surfaces should be kept clean and free of finger marks, dust, etc., by the use of a fine linen or silk wiping cloth.

TABLE II. Measurement of Opaque Areas of Metal Squares on a Transparent Circular Background of Known Area (125.10 cm²).

Opaque area on transparent circle		Galvanometer scale reading*	Opaque area, cm² Calculated	Opaque area, cm² Observed
0		100	0	0
2.54 cm opaque squares	(1)	94.8, 94.6	6.452	6.645
	(2)	89.5, 89.7, 89.6	12.90	13.03
	(3)	84 , 84.7, 84.3	19.36	19.61
	(4)	79 , 79.6, 79.1	25.81	26
	(5)	73.8, 74.3, 74.3	32.26	32.13
	(6)	68.8, 69.2, 69.3	38.71	38.52
	(7)	63.8, 63.9, 63.9	45.16	45.16
" " " "	(7)	—	48.39	48.65
1.27 " " "	(2)	61.1, 61.1		
		↓ ↑ ↓		

* Serial readings of progressively changed areas, performed three times. Decreasing and increasing of opaque areas denoted by the symbols ↓ and ↑ respectively.

TABLE III. Effect of Choice of Standard on Accuracy of Area Measurement.

Stand. transparent area and galvanometer setting	Object in light path	Galvanometer scale reading	Area, cm² Calculated	Area, cm² Observed
56.33 cm² rectangle at 100	Opaque	54.3	25.81	25.81
		65.6	19.36	19.36
		77	12.90	12.97
		88.6	6.452	6.452
19.74 cm² circle " "	"	66.9	6.452	6.536
" " " " "	Transparent	24.7	4.903	4.878
56.33 cm² rectangle " "	"	3	1.613	1.677
19.74 cm² circle " "	Opaque	91.7	1.613	1.639
6.452 cm² square " "	"	74.7	1.613	1.632
56.33 cm² rectangle " "	Transparent	.9	.403	.516
19.74 cm² circle " "	Opaque	97.7	.403	.452
6.452 cm² square " "	"	93.7	.403	.406
" " " " "	Transparent	6.2	.403	.400
*1.613 cm² " " 50	Opaque	37.4	.403	.406
*1.613 cm² " " "	Transparent	12.4	.403	.400

* With light intensity increased.

source of the areameter along its supporting column as close as possible to the base plate, with the 1.606 cm² transparent area serving as a standard set at a maximum possible reading of 50, readings of 37.4 on 4 different opaque metal 0.402 cm² squares were obtained, giving an area to within 0.0032 cm² (0.0005 in.²) of the true value (Table III).

A confirmation of the accuracy of the areameter results was obtained by other independent methods of area measurement. Irregularly shaped areas were randomly cut from cardboard and weighed against accurately cut squares of the same material. The results were not good, owing to the perceptible absorption of atmospheric moisture by the material. However, when thin .0061 cm (0.0024 in.) aluminum foil was used to make such areas, photoelectrically and gravimetrically determined values (ranging in size from 4.819 to 30.840 cm² (0.75 to 4.8 in.²) were in agreement to within one per cent (Table IV). Comparison of photoelectric with planimetric measurements (also shown in Table IV) of irregularly shaped areas having the general dimensions of electrophoretic plasma patterns, likewise showed agreement of the expected order of magnitude.

Discussion. Although the principle on which it is based is not new¶ the apparatus

¶ In the course of this work, our attention was called to a photoelectric apparatus for area measurement of plant leaves reported by Mitchell(1) and subsequently modified by the American Instrument Co. Both models were constructed of more expensive materials, utilized a different optical arrangement, and neither would be suitable for the accurate measurement of areas less than 20 cm² (3 in.²).

TABLE IV.
Comparison of Results of Area Measurements by Different Methods. Area in cm^2 measured by

Photoelectric areameter	Wt of sheet metal	Planimeter
4.807	4.848	—
19.10	19.03	—
12.13	12	—
7.007	7.039	—
23.94	23.68	—
30.91	30.97	—
11.42	—	11.61
17.42	—	17.55
17.68	—	17.68
19.61	—	19.81
16.45	—	16.58
13.23	—	13.10

and the generally outlined method for area measurement described in the foregoing have obvious advantages over other methods known to us or in use at the present time. Planimetry, whether direct or involving the tracing of enlargements of an original pattern, is time-consuming and fatiguing. A method which has been in use since the last century, namely that of weighing standardized sheet materials (2) may be highly inaccurate, as has been our experience in the use of paper or cardboard. Weighed thin sheet metal is feasible for occasional use but not practicable for any amount of work in the laboratory. Instruments such as the curve computer(3), differential analyzer(4), electro-mechanical integrator(5), and the Knorr-Albers microphotometric area determinator(6), more recently suggested for the measurement of areas in various types of physical and chemical studies, are all too expensive and too complicated for use by any but a few laboratories. Moreover, these instruments offer no advantage over the photoelectric areameter here presented, either in respect to accuracy or range and sensitivity.

The general application of area measurement as a method of quantitative analysis, appears to be increasing. In addition to the general and particular fields of chemistry, physics and biology represented by the foregoing citations, area measurements have been applied in analytical chemistry to the measurement of precipitates(7), the calibration of microburettes(8), the determination of fat as monolayer films(9), and in the quantitative estimation of α-amino acids by paper chromatography(10). Detailed methods and technics of measurements will be described in future communications from this laboratory concerned with work in electrophoresis, ultracentrifugation, and anthropometry. The simplicity, rapidity, and accuracy of the technic outlined in this paper may lead to the favorable consideration of area measurement not only for the purposes mentioned but for new uses also.

Summary. An apparatus utilizing the principle of the photoelectric measurement of intercepted light, and technics for its use in the rapid and accurate measurement of a wide range of areas are described. Application to a variety of quantitative physical, chemical, and biological measurements is indicated.

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Effect of Amino Acid Imbalance on Course of Lansing Poliomyelitis in Mice.* (19917)

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Over a period of years considerable evidence has been obtained indicating that malnutrition results in a decreased susceptibility to some viral infections(1). Jones *et al.*(2) have reported a sparing effect of low protein and low tryptophan diets on mice with Lansing poliomyelitis, and Davies *et al.*(3) have studied the influence of deficiencies of each of the essential amino acids on the course of Lansing poliomyelitis and have found tryptophan, isoleucine, valine and methionine deficiencies especially effective in suppressing the Lansing virus. Rasmussen *et al.*(4) reported that 6-methyl tryptophan exerts similar action on Lansing infections in mice. The experiments reported here were designed to compare the effect of methionine and tryptophan analogues and rations containing excessive amounts of various amino acids on the course of Lansing poliomyelitis in mice.

Methods and results. In all of these experiments, 4-5 week old Webster Swiss mice from our colony were used. Purified rations containing casein as a protein source or mixtures of amino acids replacing protein were fed. The mice were inoculated intracerebrally with a suspension of spinal cords and medullas from mice infected with Lansing poliomyelitis virus (usually 0.03 ml of a 1% suspension, containing 100 LD_{50} of virus). It has been demonstrated that methionine sulfoximine acts as a methionine antagonist for various bacterial and animal species(5-7). After preliminary toxicity studies, 70 mice in each of 2 groups were placed on a 9% casein diet. A week later all of the animals were inoculated intracerebrally with 10 LD_{50} of Lansing virus, and daily intraperitoneal injections of ½ mg of methionine sulfoximine were started. The results of this experiment indicated that methionine sulfoximine had a slight effect in prolonging the incubation time. However, in this experiment methionine sulfoximine was of little real value in altering the course of poliomyelitis in mice. These observations agree with Ainslie's(8) report on the anti-Lansing activity of methionine sulfoximine.

In another series of experiments several tryptophan analogues were tested. These included 6-methyl tryptophan for comparative purposes, 4,6-dimethyl tryptophan, 4-methyl tryptophan, 5-methyl tryptophan, alpha-ethyl tryptamine and alpha-amino (3 indazole) propionic acid. These analogues were incorporated into amino acid rations containing 0.03% and 0.05% DL tryptophan. Of these alpha-ethyl tryptamine and 5-methyl tryptophan appeared to be the most effective tryptophan antagonists so far as the nutrition of the mice was concerned; but alpha-ethyl tryptamine had no significant influence on Lansing infections and 5-methyl tryptophan even when fed at highly toxic levels did not exhibit a protective action comparable to that obtained with 6-methyl tryptophan.

Following this series with methionine and tryptophan deficiencies the effect on mouse poliomyelitis of amino acid imbalances caused by feeding excess methionine and tryptophan was studied. The results of a preliminary experiment suggested that excess methionine but not excess tryptophan was protective.

Consequently, the following experiment was undertaken. Three groups of 28 mice each

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TABLE I. Effects of Excess Methionine on Resistance of Mice to Lansing Virus.

Ration	9% C + 5% DLm*	9% C + 3% DLm	9% C + 1% DLm	9% C
No. of mice†	26	28	27	28
Survivors	0	1	0	0
Avg incubation period, days	14	12.1	7.5	7.1
Avg survival time, days	14.9	13.3	8.5	8.5

* C = Casein; DLm = DL methionine.
† No. of mice in each group alive at time of inoculation.

TABLE II. Effects of 5% DL Methionine Ration on Resistance of Mice to Lansing Virus.

	Group			
	Mice on ration 1 wk prior to inoc.	Mice fed ration 2 days, 9% casein 1 day	Mice on ration 1 day prior to inoc.	9% casein control
No. of mice*	26	27	28	28
Survivors	0	0	1	0
Avg incubation period, days	14	8.4	9.2	7.1
Avg survival time, days	14.9	9.7	11.2	8.5

* No. of mice in each group alive at time of inoculation.

TABLE III. Effect of 6-Methyl Tryptophan + Methionine on Resistance of Mice to Lansing Virus.

Ration	.4% 6-MT + 3% DLm*	.4% 6-MT	3% DLm	Control
No. of mice	14	14	14	14
Survivors	2	0	0	0
Avg incubation time, days	14.3	9.6	9.9	7.9
Avg survival time, days	17.5	11.4	11.1	9.1

* DLm = DL methionine.

were placed on 9% casein rations containing 1, 3, and 5% DL methionine one week before intracerebral inoculation with 100 LD_{50} of Lansing virus. Another group of 28 mice was fed alternately a 9% casein diet for one day followed by 2 days of a ration containing 9% casein + 5% DL methionine. A fifth group was placed on a 9% casein diet for 6 days and then changed to the 9% casein, 5% DL methionine ration the day preceding inoculation. A control group was fed the 9% casein diet but no excess methionine. Uninoculated nutritional controls were also set up for each of these 6 groups. The experiment was terminated 28 days after inoculation, and the results obtained are shown in Tables I and II.

It can be seen from Table I that rations containing 9% casein, when supplemented with 3 or 5% DL methionine, delay the onset of symptoms in mice intracerebrally infected with Lansing virus. Table II indicates that in order fully to obain this effect, a continuous period of high methionine intake is necessary prior to inoculation.

Similar experiments with excess leucine, lysine, histidine, phenylalanine and choline, a methyl donor, were negative.

To determine whether 6-methyl tryptophan and excess methionine were synergists in their protective action, 4 groups of 14 mice each were placed on amino acid mix rations containing 0.05% DL tryptophan one week before being intracerebrally inoculated with 100 LD_{50} of Lansing virus. The ration fed the first group was supplemented with 3% DL methionine. The second group's ration was supplemented with 0.4% 6-methyl tryptophan. The third group had 3% DL methionine and 0.4% 6-methyl tryptophan added to its ration, and the fourth group was used as a control. The results obtained in this experiment are shown in Table III.

These data show that a combination of 0.4% 6-methyl tryptophan and 3% methionine exerts a greater protective effect against Lansing poliomyelitis in mice than was obtained with these levels of either compound alone.

Discussion. The decreased susceptibility of mice to Lansing virus brought about by the feeding of 6-methyl tryptophan and excess methionine is manifested as essentially a delay in the onset of symptoms with a resultant prolonged survival time. In experiments employing 6-methyl tryptophan at higher levels without excess methionine a significant proportion of mice are protected completely(4). A very low survival rate has always been obtained when excess methionine has been fed

alone. This has been due in part to the toxicity and lack of palatability of high methionine rations. Uninoculated nutritional controls fed high methionine rations lose about 40% of their body weight during the test period and deaths in these groups are not infrequent. However, restricted food intake experiments have shown that the protective effect of excess methionine is not directly related to starvation.

Summary. 1. A decreased susceptibility to Lansing poliomyelitis, characterized by prolonged incubation and survival times, has been observed in mice fed excess methionine. To obtain this effect fully, a continuous period of high methionine intake was necessary prior to inoculation. 2. The addition of excess methionine to low tryptophan rations containing 6-methyl tryptophan resulted in a more marked protection against Lansing infection in mice than when the same amounts of methionine or 6-methyl tryptophan were fed alone.

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Studies on Irradiated Animals, III. Effect of Saline on Radiation-Induced Mortality and Weight Changes.* (19918)

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In pharmacological studies it is customary to dissolve chemicals to be injected in saline solution, and also to use saline as control. However, in pharmacological studies on irradiated animals saline solutions may not be inert since the "acute radiation syndrome" includes disturbances of the salt-water metabolism(1). Saline injections might, therefore, alter the course of radiation death. In order to clarify the effect of physiological sodium chloride injections on irradiated animals, the investigations reported in this paper have been undertaken.

Materials and methods. Swiss, male white mice of the Institute strain, 22 g ± 15% of body weight, were used. Irradiation was done as described previously(2,3). The radiation factors were: 200 kv, 15 ma, .25 Cu ± 1.0 mm Al filtration, HVL = .8 mm Cu. X-ray doses of 410 and 385/air, representing the LD_{60}/14 days and LD_{30}/14 days were given. Saline was administered daily in amounts of .3 cc of a 0.9% NaCl solution either intramuscularly or intraperitoneally, starting immediately after exposure, for an over-all period of 14 days (with the exception of Sundays) or for 6 consecutive days.† For graphic presentation of results the mortality rate of saline-injected animals is compared with that of the non-medicated irradiation control group. The figures in parentheses indicate the number of animals in the different groups.

Results. 1. *Effect of intramuscular saline injection in its relationship to the time of saline administration.* Fig. 1 summarizes pertinent results obtained in this study. As indicated in Fig. 1, saline injection slowed the death rate at the end of the first week for 2 or 3 days. If injection of saline was continued over the 14-day period (curve B), then a

*The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

† The animals were kept during the 28-day observation period in air conditioned quarters at 75°F.

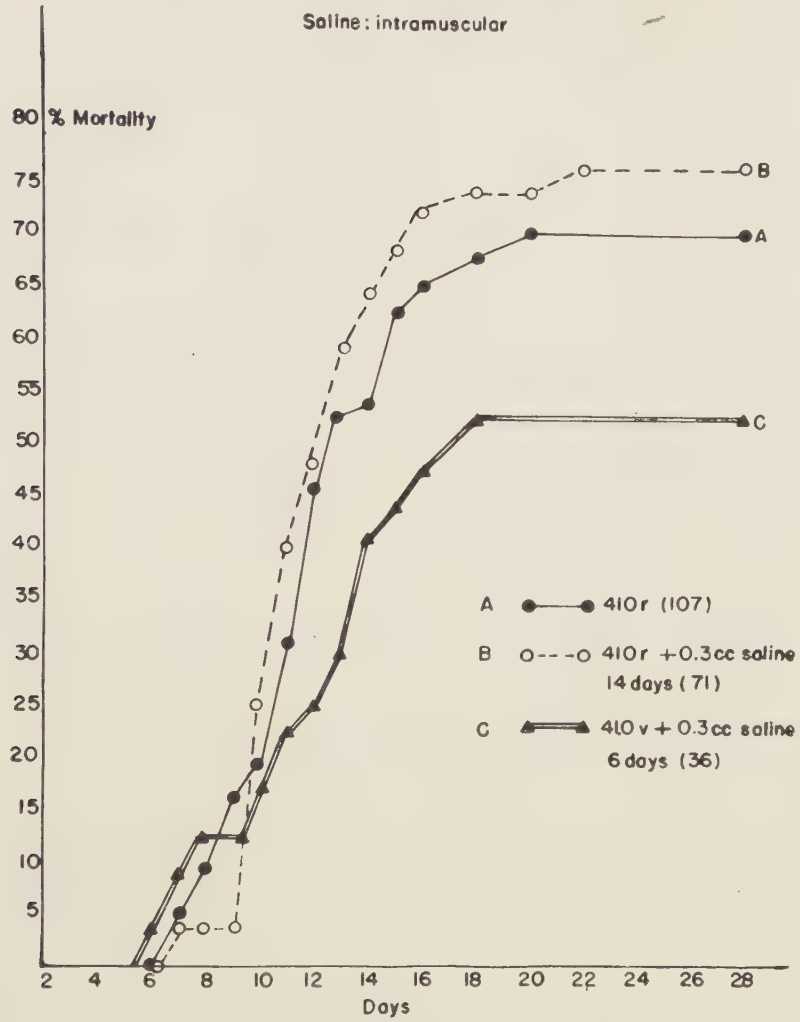

FIG. 1. Influence of length of inj. time on radiation-induced mortality: The graph demonstrates that reducing the over-all inj. time of saline from 14 to 6 days produces a statistically significant decrease in mortality.

steep increase of mortality took placed around the 11th day and the mortality curve of animals treated in this manner showed a slight, even though statistically not significant, increase. The mortality curve of animals receiving saline injections for 6 days only (curve C) indicated a statistically‡ significant decrease. (Comparison of curves B and C: $P < .01$ for the 16th day using the χ^2 method.) The delay in the development of mortality in saline-treated animals is also noticed in those animals exposed to the lower radiation dose as demonstrated in Fig. 2. There is also a slight, but not significant, decrease in the mortality rate of the saline-treated animals.

2. *Comparison of intramuscular vs. intraperitoneal injections.* In both instances administration of saline took place over a 14-day period and was administered to mice exposed to the X-ray dose of 410 r. There was no significant difference between the 2 mortality curves. But attention should be called to the fact that a delay of death for a period of 3

‡ We are indebted to Dr. E. A. Jerome for the statistical analyses.

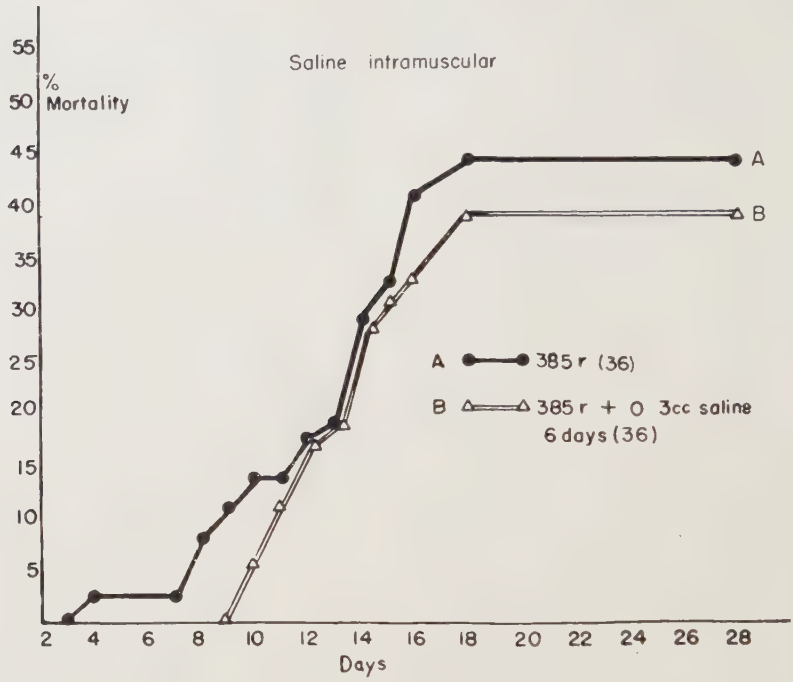

FIG. 2. Effect of 6-day inj. of saline on a lower X-ray mortality: At this level of X-ray mortality, saline inj. produces only slight delay in the development of mortality.

days at the end of the first week was also noticed in the intraperitoneal group.

3. *Influence of saline injections on radiation-induced weight changes.* The influence of saline injections demonstrated in the study of mortality is reflected in a similar manner in the weight curves of the animals as shown in Fig. 3.

Discussion. The results obtained in this study clearly indicate that saline cannot be considered as an inert agent in pharmacological studies on irradiated animals. This has to be kept in mind if and when the effect of a certain chemical dissolved in saline is used for a study of its effect on radiation-induced mortality.

Attention is called to the fact that saline administration *subsequent* to the exposure to lethal X-ray doses may under certain conditions change the mortality rate.

Furthermore, it appears noteworthy that the pharmacologically induced change of radiation mortality is reflected in the weight curve of the animals. As indicated in Fig. 3, weight gain in mice after saline administration for 6 days, which resulted in a significant reduction in mortality, was more speedy in this than in the non-medicated irradiated group or in the irradiated group of mice receiving saline over the 14-day period, which procedure slightly increased mortality. This observation is in line with our previous findings concerning the correlation between weight changes and percentage mortality as produced by varying X-ray doses(3).

Summary. 1. Daily injections of .3 cc of .9% NaCl solution into mice of 22 g $\pm$ 15% body weight subsequent to the exposure to X-ray doses representing the $LD_{60}/14$ days and $LD_{30}/14$ days for periods of 14 and 6 days respectively caused a delay in the development of the mortality produced by these X-ray doses. 2. While the 14-day administration of saline produced a slight but statistically not significant increase of the X-ray-induced mortality rate, the 6-day administration resulted in a statistically significant decrease of the mortality rate in mice exposed to the $LD_{60}/14$ days. 3. The saline-induced change of X-ray mortality was also reflected

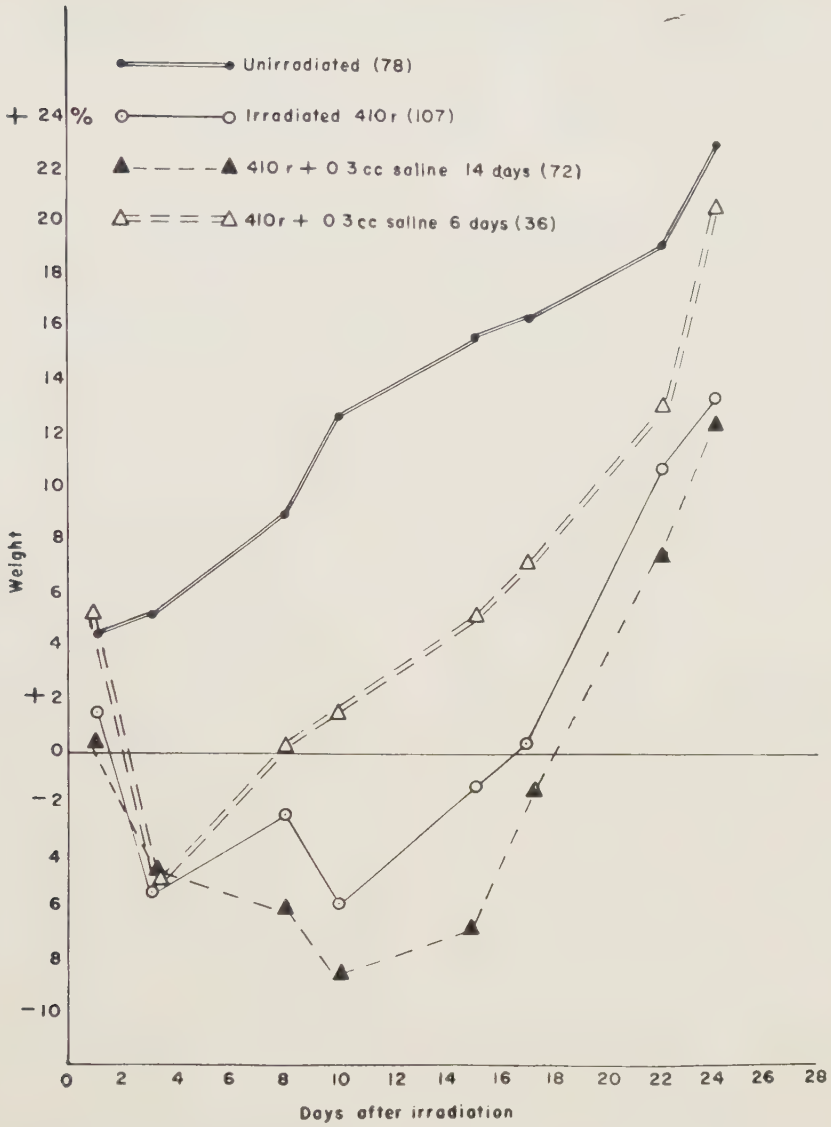

FIG. 3. Influence of intramusc. saline inj. on radiation-induced wt changes: In general the wt curves reflect the changes produced by 6-day saline administration on mortality. The wt gain in mice is more speedy in the group receiving saline for 6 days than in either the non-medicated irradiated group or the irradiated group of mice receiving saline for 14 days.

in the weight curve of the animals.

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Distribution of Radioactivity Following Intravenous Administration of Trivalent Chromium 51 in the Rat and Rabbit.* (19919)

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The use of chromium^{51} as a biological tracer has recently been reported by Gray and Sterling(1). They suggested that the hexavalent form was taken up by the red cells and the trivalent form combined with plasma proteins *in vivo* and *in vitro*. In view of the possible application of chromium^{51} labelled proteins to biological and medical studies the uptake and distribution of trivalent chromium^{51} alone must be established in order to interpret these studies. Since trivalent chromium is relatively insoluble at the pH of blood, and readily forms colloid complexes, it seemed of interest to determine whether the distribution of radioactivity after administration of trivalent chromium^{51} would be similar to that of other colloids.

Materials and methods. The Cr^{51} was obtained from Oak Ridge and the Abbott Laboratories as the chloride with a specific activity of approx. 100 μc per mg. For injection, an aliquot of this solution was added to isotonic acetate buffer at pH 5.5. Twenty-four male rats, weighing between 180-200 g were used for the first set of experiments. Each animal received 0.2 mg of chromium containing approx. 20 μc of Cr^{51} via caudal vein injection. The animals were sacrificed by exsanguination under anaesthesia at time intervals up to 24 hours. An *additional experiment* was carried out utilizing 2 male rabbits weighing approx. 2 kilos each. The rabbits received, intravenously, 2.0 mg of chromium containing approx. 200 μc of Cr^{51}. One rabbit was sacrificed 12 hours after injection, the other at 24 hours. Of the *tissues* studied, weighed representative portions were placed in vials and digested with a constant volume of 10% NaOH, while bone samples were digested in concentrated HNO_3. Diluted standards of the injected material were prepared in similar vials. Radioactive determinations were obtained by the use of a cup-type torroidal gamma counter.‡ In order to determine *sites of localization* in the bone, the tibia of each rabbit was prepared for radioautography. The bone was frozen in liquid air, sawed longitudinally with a carborundum wafer, and placed on no-screen X-Ray film for 7 days. In order to determine whether the radioactivity was bound to protein in the plasma and various tissues, the proteins were precipitated from the plasma and saline extracts of the liver and spleen homogenates with 10% TCA. Marrow was removed from a portion of the femur, suspended in 10 volumes of isotonic saline, and centrifuged to remove the cellular components. 10% TCA was then used to precipitate the protein dissolved in the saline. The fatty material obtained by centrifuging the marrow suspension was extracted with a 3:1 alcohol-ether mixture.

Results. The results of the rat experiment are summarized in Table I. At the end of the first hour the radioactivity (expressed as % of injected dose per g of tissue) of the bone and liver approximated that of the blood. The highest concentration of Cr^{51} was found in the kidney while the spleen and muscle showed negligible uptake. During the ensuing 24 hours, the Cr^{51} was lost rather rapidly from the blood and liver, and decreased somewhat in the kidney. The bone, however, showed considerable retention, and at the end of 24 hours contained the major portion of the remaining radioactivity. The concentration in the kidney is a reflection of the rapid excretion of part of the injected chromium for 40% of the total activity was found in the urine at the end of 24 hours. No activity was noted in the feces. Only after 24 hours did the concentration of Cr^{51} in the spleen of the rat approach that of blood.

Extension of these studies to determine the sites of localization of radioactivity in the bone showed most of the Cr^{51} to be concen-

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† Pre-doctoral Fellow, National Science Foundation.

‡ Furnished through the courtesy of the Texas Co.

TABLE I. Radioactivity of Tissues of Rats after Injection of $Cr^{51}Cl_3$.

Time after inj., hr	% of inj. dose/g of tissue. Each value represents avg of 4 animals ± stand. error					
	Blood	Bone	Spleen	Liver	Kidney	Muscle
1	1 ± .08	1 ± .11	.28 ± .01	1 ± .10	1.60 ± .10	.10 ± .0
2	1.27 ± .11	1.57 ± .18	.30 ± .14	1.10 ± .10	1.70 ± .10	.09 ± .01
4	1.23 ± .10	1.65 ± .07	.40 ± .10	.80 ± .14	1.65 ± .18	.09 ± .01
6	.57 ± .05	1.39 ± .04	.18 ± .01	.68 ± .13	1.03 ± .19	.06 ± .01
12	.54 ± .03	1.30 ± .01	.26 ± .03	.50 ± .04	1 ± .17	.07 ± .01
24	.34 ± .02	1.70 ± .08	.40 ± .10	.48 ± .04	1.18 ± .16	.08 ± .01

TABLE II. Radioactivity of Tissues of Rabbit after Injection of $Cr^{51}Cl_3$.

Time after inj., hr	% of inj. dose/g of tissue. Each value represents one animal							
	Blood	Bone	Bone without marrow	Marrow	Spleen	Liver	Kidney	Muscle
12	.15	.13	.08	.25	.35	.14	.14	.01
24	.07	.22	.16	.41	.25	.17	.08	.003

trated in the marrow. Since complete removal of the marrow in the rat femur and tibia was difficult, a similar experiment was performed using the rabbit. In the larger animal removal of the marrow was more easily accomplished and large distinct radioautographs of split long bones could be obtained.

The uptake by the various tissues of the rabbit is summarized in Table II. The highest concentration of Cr^{51} of the rabbit tissues studied was found in the spleen, which is in marked contrast to the findings of the rat. The apparent differences between the other values for the rat and the rabbit are due primarily to a difference in total blood volume in relation to organ size and body mass. Most of the Cr^{51} taken up by the intact bone of the rabbit was concentrated in the marrow. This was borne out by a radioautograph of a split tibia (Fig. 1) which showed also an additional concentration of activity in the region of the epiphysial plate.

Studies were undertaken to determine whether or not the Cr^{51} was bound to protein. For this purpose tests were made on the blood serum, liver, spleen, and bone marrow of a rabbit killed 24 hours after injection with radio-chromium. In the plasma, 80-90% of the radioactivity was found to be associated with the TCA precipitate. Paper strip electrophoresis of this plasma at a pH of 8.6 showed Cr^{51} activity associated with all the serum proteins, but in addition to this, a region of non-protein-associated activity was found migrating toward the cathode. Relative to protein bound Cr^{51} in liver and spleen, it was found that the saline extracts of homogenates of these organs contained only 40% of their total radioactivity, and 80-90% of this was precipitable with TCA.

Centrifugation of a suspension of the bone marrow in saline and subsequent determination of radioactivity of the sediment, supernatant, and the top fatty layer revealed that the supernatant and the insoluble fatty layer contained almost all of the activity with only a negligible amount associated with the cellular elements. TCA precipitation of the saline supernatant demonstrated that the Cr^{51} remained with the protein precipitate. After treating the insoluble fatty layer with Bloors solution (3:1 alcohol-ether mixture) to remove the lipids, the radioactivity was retained in the residue. Microscopic examination of marrow smears did not reveal the presence of particulate matter in the macrophages.

Discussion. Chromium, when administered intravenously as $CrCl_3$, in addition to its retention in the plasma, is taken up by the reticulo-endothelial system, primarily the bone marrow. This is shown by the fact that in both the rat and rabbit, the marrow, 24 hours after injection, contains more of the injected Cr^{51} than does any other organ or tissue, not only in total activity but also in activity per g of wet tissue.

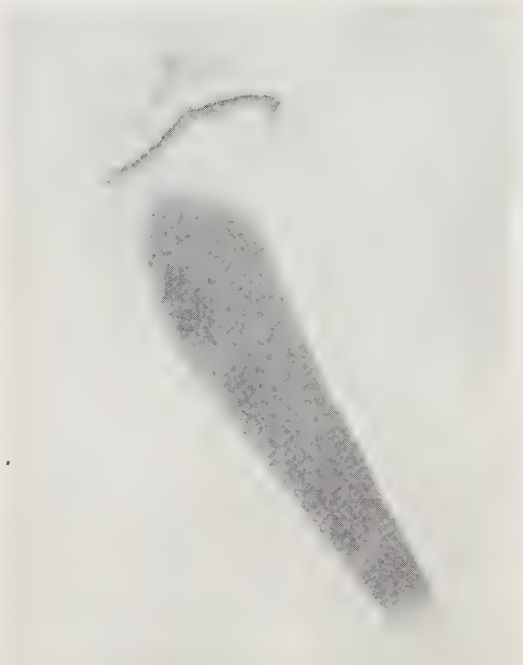

FIG. 1. Radioautograph of split tibia of rabbit 24 hr after administration of Cr^{51}.

The distribution of Cr^{51} throughout the various tissues of these animals resembles the distribution of various radioactive colloids, particularly Yttrium and Zirconium colloids (2,3). Selective localization of colloids in the reticulo-endothelial system is greatly dependent on the particulate size of the colloid. Small size particles localize to a great extent in the bone marrow and spleen. In view of the physical characteristics of the trivalent chromium, these experiments suggest that the chromium might form a small size colloid as indicated by the localization sites. However, we have been unable to demonstrate the actual presence of such colloidal aggregates or particles in the tissues or the serum by ordinary methods.

Since these experiments indicated that much of the chromium was bound to protein in the spleen, liver and bone marrow, it is suggested that the chromium is removed by these tissues and bound to protein located in these sites. It has been shown that trivalent chromium will bind to a variety of serum proteins *in vitro* (1). Some pituitary protein preparations have also been combined with chromium *in vitro* (4), therefore it would not seem unlikely that there would be *in vivo* tagging of tissue proteins as well as those in serum.

In addition, these experiments indicate that the Cr^{51} in the bone marrow is not in the red cell precursors and that the membranes of these cells are impermeable to trivalent chromium as are those of mature red cells.

Summary. Using Cr^{51} as the tracer, chromic chloride was administered intravenously to rats and rabbits for distribution studies. At the end of 24 hours up to 40% was excreted by the kidneys, while the greatest concentration of Cr^{51} occurred in the bone marrow. It is suggested that in addition to binding with plasma proteins, the trivalent chromium deposits in the reticulo-endothelial system in a pattern similar to that of small size colloids of a slow clearing nature. The Cr^{51} in the sites of localization appeared to be bound to protein.

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Effect of Bioflavones upon Metabolism of Iodine and Iodinated Compounds in the Rat.* (19920)

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During studies on the effect of various "Vitamin P" compounds upon the development of transmissible tumors in rats, it was observed that one of these substances, hesperidine methyl chalcone, was capable of changing the normal distribution and excretion of iodide and diiodotyrosine, using I^{131} as a tag. This report is a summary of our studies of the effect of this compound and other bioflavones upon the fate of I^{131} and I^{131}-tagged tyrosine in rats.

Methods. Adult rats of both sexes, weighing from 190 to 250 g, were used as experimental animals; they were of the Long-Evans, Curtis-Dunning, or Slonaker strains. The I^{131} was obtained from the Oak Ridge National Laboratory. The diiodoI^{131}tyrosine employed was tagged with radioiodine, using the method previously reported by one of us(1). Both the radioiodine and diiodoI^{131}tyrosine were administered to the rats via the external jugular. After the administration of one or the other of these substances, animals other than controls were given varying amounts of hesperidine methyl chalcone or other bioflavones. They were administered as a saline solution by the intragastric or subcutaneous routes. Radioactive assays of the I^{131} were made with a scintillation counter composed of a thallium-activated sodium iodide crystal and an R.C.A. 5819 photomultiplier tube. The sensitivity of this counter was such that 8.5% of all of the disintegrations occurring in the material taken as a sample were observed as counts. Approximately one μc of I^{131} was administered to each animal, this amount being in the tracer dose level. The data are expressed as the per cent of the tagged iodine administered present in wet weight tissue or excreta. In the case of the diiodoI^{131}tyrosine, 2 μg were administered along with the I^{131} tag to each animal studied with this compound. Whatman No. 1 filter paper was used in the paper chromatograms.

Results. In experiments in which the inorganic iodide pool of the rats was labeled using I^{131}, it was found that the intragastric administration of H.M.C. in doses from 40 to 1,000 mg per kilo was effective in increasing the uptake of I^{131} by the thyroid, skin, and stomach plus its contents, so that the values observed were significantly greater than those

TABLE I. Fate of I^{131} in the Rat 15 Hr after Its Intravenous Administration. Animals other than controls received hesperidine methyl chalcone by the intragastric route at the same time. Data are expressed as % of I^{131} given per g of blood, liver, kidney, and skin, wet wt, and % per organ for thyroid and stomach plus contents. Five rats in each group.

		mg H.M.C. administered		
	Control	10	50	250
Blood	.11 ± .03*	.17 ± .04	.23 ± .08	.25 ± .03
Liver	.11 ± .02	.12 ± .02	.16 ± .04	.18 ± .02
Kidney	.08 ± .01	.12 ± .02	.22 ± .08	.18 ± .03
Stomach	1.25 ± .27	3.6 ± .11	3.5 ± .45	3.5 ± .8
Skin	.19 ± .01	.34 ± .09	.31 ± .08	.29 ± .04
Thyroid	16 ±1.2	25 ±2.1	20 ±3.5	21 ±1.9
Remains	.01	.01	.04	.02
Urine	67	53	44	48

* All values are means ± stand. error = $\sqrt{\frac{\Sigma \text{ dev}^2}{(N)\ (N-1)}}$.

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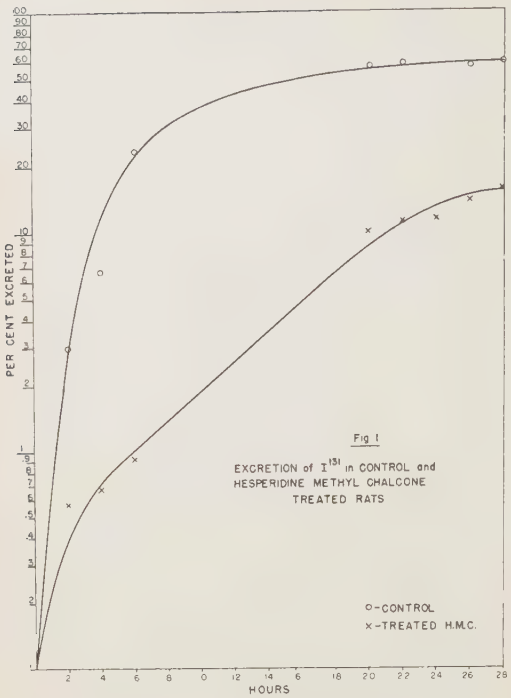
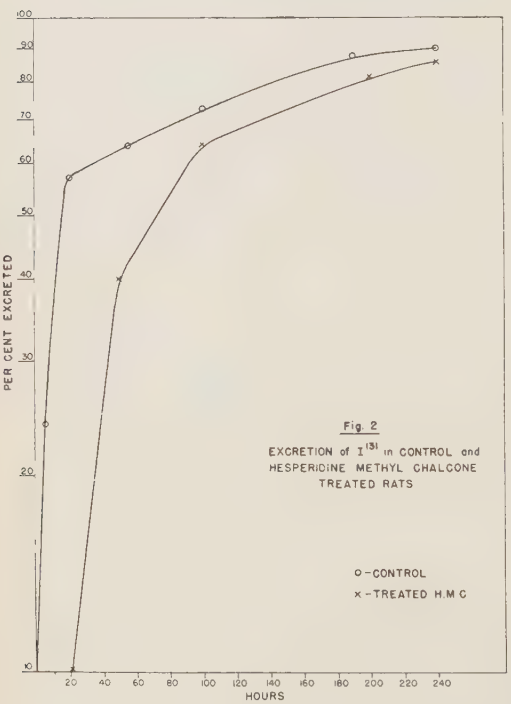




observed in the controls (Table I). A depression of the excretion of the I^{131} was observed in the H.M.C.-treated animals, as can be seen from Table I. Additional data for other tissues are also shown in Table I. When H.M.C. was given as a subcutaneous injection at the same time that I^{131} was administered intravenously, an even greater depression of the urinary excretion of I^{131} was observed. Urine samples taken at various time periods up to 240 hours after the administration of the drug and the radioactive tag showed that the urinary excretion of I^{131} was markedly diminished during the first 24 hours, as shown in Fig. 1. Following this time period, the treated animals exhibited I^{131} excretion rates which were similar to those of the controls. The relative excretion rates of I^{131} by the control and H.M.C. animals throughout the entire time period studied are shown in Fig. 2. The excretion curves presented suggest that the H.M.C.-treated animals did not catch up with the controls at time periods as long as 240 hours after the initial tagging of the iodide pool with I^{131}. The lag in excretion of the H.M.C.-treated animals resulted in an increase of the I^{131} content of thyroid, skin, and balance which was 185, 375, and 200% greater than normal respectively, 240 hours after the administration of I^{131}.

When 5 mg of sodium iodide was administered to rats as a carrier along with the I^{131} which was essentially carrier free, the depression of iodine excretion as observed with I^{131} was still obtained. For example, at 2, 5, 8, 24, 32, and 48 hours after the administration of I^{131} plus carrier, the controls excreted an accumulated total of 11, 32, 42, 87, 91, and 95% of the dose, respectively. When rats were given 80 mg of the H.M.C. subcutaneously, the excretion was found to be 1.4, 5, 5.4, 83 and 96% respectively for the time periods stated above.

The repeated administration of H.M.C. at 12- or 24-hour intervals did not depress the excretion of I^{131} indefinitely. These data suggest that hesperidine methyl chalcone can effect only a transitory depression of iodide excretion.

When diiodoI^{131}tyrosine was administered to rats which were given H.M.C. subcutaneously at dosage levels shown in Table II, a depression of urinary excretion of the I^{131} tag was observed which was similar to that

TABLE II. Fate of DiiodoI^{131}tyrosine in Rats 15 Hr after i.v. Administration. Rats other than controls received hesperidine methyl chalcone subcut. at the same time. Values are given as % of dose per g wet wt of all tissues except thyroid and stomach; these are reported as % per organ.

	Control	mg of H.M.C. per rat				
		10	20	50	80	240
No. of rats in group	30	5	5	5	5	5
Blood	.08 ± .01	.36 ± .09	.44 ± .05	.42 ± .21	.25 ± .04	.21 ± .03
Liver	.08 ± .01	.28 ± .05	.34 ± .04	.37 ± .04	.32 ± .02	.33 ± .09
Kidney	.17 ± .02	.39 ± .03	.54 ± .06	.67 ± .03	.55 ± .03	.48 ± .05
Stomach + cont.	1.7 ± .71	3.1 ± .48	7.3 ±1.1	6.5 ±1.3	12 ± .25	29 ±3.9
Skin	.17 ± .02	.59 ± .03	.72 ± .06	.91 ± .20	.81 ± .11	.55 ± .05
Thyroid	12 ± .61	25 ±1.4	14 ±1.7	13 ±2.2	8.8 ±1	4.9 ± .05
Remains*	.012± .0003	.04	.04	.04	.05	.04
Urine	69.6 ± .45	10.5	9.5	5	7.8	3.5

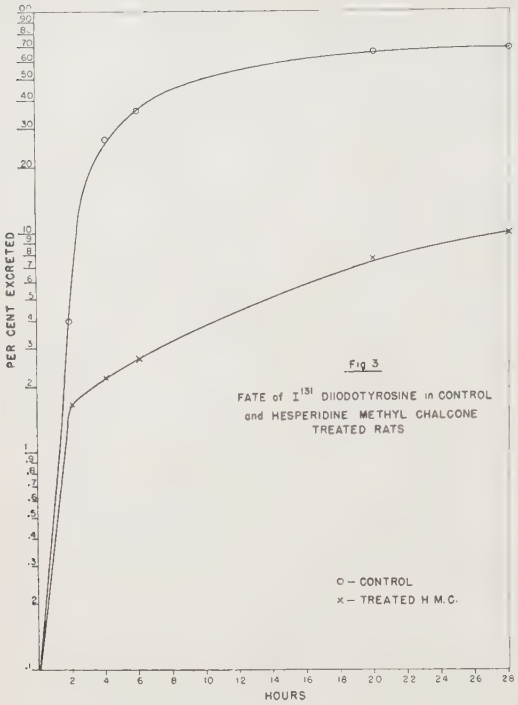
* In groups receiving H.M.C., remains and urine were measured for the group and not separately. Hence, statistical evaluation was not possible.

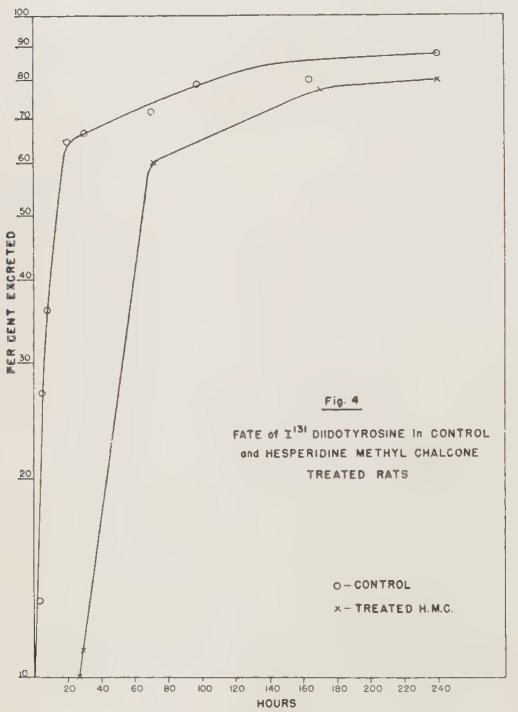
seen with inorganic iodide. The thyroid uptake of the diiodoI^{131}tyrosine was almost twice as great as normal in the group receiving 10 mg of H.M.C., whereas the higher dose levels showed only slightly less than normal uptake and finally, at the highest dose level, the thyroid uptake was depressed. A maximum uptake for skin was observed at the 50 mg dose level, although the H.M.C.-treated animals showed an uptake which was greater than normal at all dose levels. Hesperidine methyl chalcone caused an increase in the I^{131} tag of diiodotyrosine which was found in the stomach and its contents. The increase observed above normal appeared to be directly related to the amount of the drug given. The diiodoI^{131}tyrosine levels of other tissues were significantly increased when H.M.C. was administered, as shown in Table II.

Rats which were given 80 mg of H.M.C. in addition to diiodoI^{131}tyrosine and sacrificed 240 hours later showed a preliminary depression of excretion during the early time periods (Fig. 3 and 4). The tissue of these animals when sacrificed contained more of the original tag than the controls. This effect was reflected primarily by higher uptakes which were observed in thyroid and skin. The thyroids of the controls contained 2 ± .82% of the dose, whereas the thyroids of the H.M.C.-treated rats contained 3.4 ± .61% of the dose. The difference between these 2 groups appears to be significant. A comparison of the means of the 2 groups gave a p value of less than .01.

In an effort to gain additional information concerning the mechanism whereby H.M.C. reduces the excretion of iodide, studies were made upon the relative concentrations of I^{131} present in the red cells of normal and H.M.C.-treated rats; this result was compared to the I^{131} plasma concentration. As shown by one of us(2), this test may be employed to determine the degree of binding of iodide into a molecule which may be too large to penetrate the red cell membrane. Thus the ratio of the iodide space in the red cell of the rat compared to that of plasma when all of the I^{131} is present as iodide, was found to be 0.76 ± 7% in the control animals used. Similar measurements upon blood samples drawn from rats previously treated with 80 mg of H.M.C. gave ratios of 0.74 ± 7%. These results indicated that the H.M.C. did not bind the inorganic iodine into a complex which was too large to penetrate the red cell membrane. The greatly reduced urinary excretion which was observed can not be explained by virtue of the fact that the iodide is bound in a molecule which is too large to escape filtration through the glomerular membrane.

One dimension water chromatograms of varying amounts of iodide and hesperidine methyl chalcone revealed that these 2 substances appear to combine in water solutions. Using I^{131} as a tag, the movement on paper for iodide differed from that of hesperidine methyl chalcone plus iodide. An examination of the chromatogram with ultraviolet light revealed that iodide plus H.M.C. formed





fluorescent compounds which had R.F. values which differed from that seen for H.M.C.

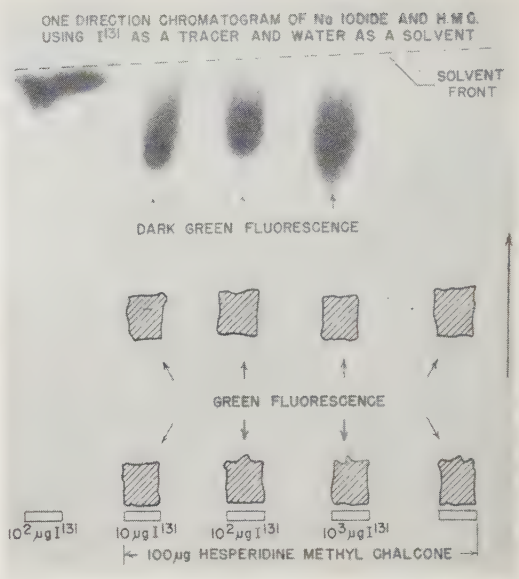


FIG. 5.

alone. The chromatogram is shown in Fig. 5.

Studies with other bioflavones and related compounds, such as sodium hesperidine chalcone, eriodictin, and sodium rutin revealed that these substances were able to influence the metabolic pattern of a tagged dose of I^{131} in a manner similar to that observed with H.M.C. These data are summarized in Table III.

It has been postulated by Parrot *et al.*(3), and Wilson *et al.*(4) that the bioflavones exert a sparing action upon epinephrine in the body. In these studies it was found that the administration of ephedrine, which blocks the destruction of epinephrine by amine oxidase(5), affected the fate of diiodoI^{131}tyrosine in a manner similar to H.M.C. For example, rats given 50 mg of ephedrine in 6 ml of saline solution subcutaneously, along with an intravenous dose of diiodoI^{131}tyrosine, and sacrificed 15 hours later showed a urinary excretion of the I^{131} tag which was only 12.2% of normal. Values for skin, thyroid, and stomach plus contents were observed to be 300, 75, and 1500% of normal respectively. When epinephrine itself was administered to rats, a reduction of the excretion of the I^{131} tag of diiodoI^{131}tyrosine was observed and was 48% of normal. Values for skin, thyroid, and stomach plus contents were observed to be

TABLE III. Distribution of I^{131} 15 Hr after Intrav. Administration in Rats Treated with Various Flavones. Values given are presented as % of uptake observed in normals. 80 mg of the flavone listed were given to each rat in 6 ml of solution. Five animals in each group.

	Rutin	Hesperidine chalcone	Eriodictin
Skin	415	415	505
Thyroid	91	74	74
Stomach	425	920	670
Urine	28.5	17	21

230, 44, and 830% of normal respectively.

Discussion. These studies show that the bioflavone derivative, hesperidine methyl chalcone, is able to depress the excretion of iodide by the rat. The administration of a dose of carrier sodium iodide, which was approximately 2,500 times greater than the normal total thyroid iodide content(6), with the I^{131} gave similar results. Hesperidine methyl chalcone was not able to block the excretion of iodide indefinitely, since repeated doses showed no greater effect upon iodide excretion than one single dose. Presumably, because of the depression of excretion of iodide, an increased uptake of I^{131} by the thyroid and skin was observed when H.M.C. was given orally. No essential difference was observed between doses of 10 mg or 250 mg. These data suggest that only small amounts of H.M.C. are absorbed by the intestinal tract. Paper chromatograms of iodide and H.M.C. suggest that bioflavones alter the metabolic pattern of iodide in the body by acting as an iodine "trap".

Summary. Studies with bioflavones such as hesperidine methyl chalcone showed that these compounds are capable of altering the thyroidal accumulation of I^{131} and of diiodoI^{131}-tyrosine. The intragastric administration of various amounts of H.M.C. caused an increased uptake of I^{131} or I^{131}-tagged material by the thyroid and skin. A reduction in the I^{131} excreted was observed. When H.M.C. was administered subcutaneously, the excretion of I^{131} was even more markedly reduced. Studies with other bioflavones such as sodium hesperidine chalcone, eriodictin, and sodium rutin showed these compounds to effect iodine metabolism in the same manner as H.M.C. These studies suggest that the effects observed with H.M.C. upon iodine metabolism may be related to its epinephrine sparing action when present in small amounts. The administration of large amounts of H.M.C. depresses thyroidal accumulation of I^{131}. Paper chromatograms indicate that larger amounts of H.M.C. in the body act as an iodide "trap" and thereby prevent the accumulation of I^{131} by the thyroid.

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Influence of Hemagglutinating Viruses on Tumor Cell Suspensions: II. Newcastle Disease Virus and Ehrlich Carcinoma.* (19921)

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In a previous paper the inhibitory effect of Newcastle Disease virus (NDV) on the growth of cell suspensions of sarcoma 180 was reported(1). When equal parts of virus and cell suspension were mixed at 4° and room temperature (25°) immediate inoculation of the mixtures failed to result in tumor growth. When the same mixtures were kept at 4° and then transferred to room temperature, or if they were kept continuously at room temperature for one to 3 hours, their inoculation resulted in tumor growth.

It has been noted that NDV also has the power of acting on the cells of the Ehrlich carcinoma(1). By using this tumor, which produces ascites in mice, it was possible to compare the *in vivo* and *in vitro* action of NDV on the tumor cell. These studies are now presented. Since this work was completed Kausche, Landschutz and Sauthoff(2) have reported the effect of PR8 strain of influenza virus on the Ehrlich carcinoma. Our results with NDV have been similar to theirs and will be discussed later.

Materials and methods. Virus. The Massachusetts strain of NDV† was prepared by inoculation of 0.2 cc of a 10^{-3} dilution into 11-day fertile eggs and the clear allantoic fluid harvested 48 hours later. Pools were kept frozen until used. Normal allantoic fluid (NAF) pools were made in the same manner from uninoculated eggs. *Titrations.* Hemagglutination (HA) titrations were done by diluting the material to be tested in saline to make dilutions of 1-10, 1-100 and doubling dilutions thereafter. Equal quantities (0.5 cc) of ¼% washed adult chicken red blood cells were added and the tests were read after one hour at room temperature. The last tube showing complete hemagglutination was taken as the HA titer. Titrations in eggs were done by inoculating 0.2 cc of virus dilutions into the allantoic sac in groups of 3 to 5, 10-11-day-old fertile eggs. Embryo death was taken as the end point and the titer calculated according to the method of Reed and Muench(3). *Tumor suspensions.* Mice bearing Ehrlich carcinoma which had been implanted subcutaneously 10 to 14 days previously were killed and their tumors removed aseptically. The tissue was pushed through a 40-mesh monel metal screen with a pestle and made to 10% suspension in buffered Locke-Ringer (pH 7.2) to which 0.6% glucose had been added. After settling, a fairly uniform single cell suspension was obtained. For long experiments 100 units penicilllin and 2.5 mg streptomycin per cc were added.

Results. In vitro experiments. The general

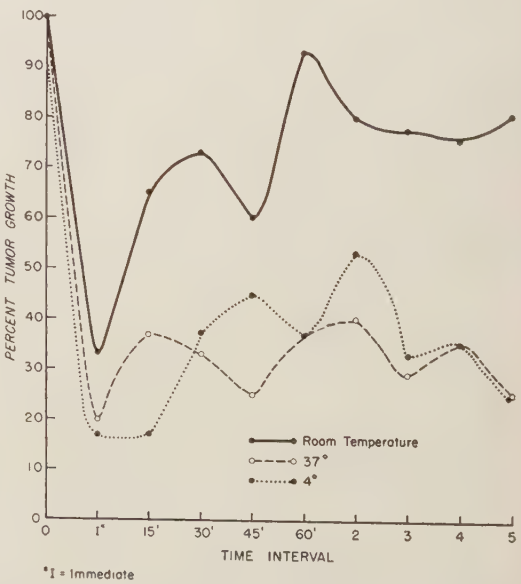

FIG. 1. Percentage growth of NDV and Ehrlich carcinoma cell suspensions mixed at 4°, 25° and 37° and inoculated subcut. at different time intervals.

* This work was supported by funds from the National Cancer Institute of the U. S. Public Health Service, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

† Obtained through the courtesy of Dr. George Sharpless of Lederle Laboratories.

plan of experiments was the same as has been reported previously(1). Equal amounts of tumor suspension (10%) were mixed with equal amounts of undiluted NDV allantoic fluid. At different time intervals and at different temperatures 0.2 cc of the mixtures were inoculated subcutaneously into each flank of groups of 5 white mice. At the same time 1.0 cc of the mixture was rapidly sedimented in an angle-head centrifuge and a HA titration done on the supernatant. The animals were inspected at weekly intervals for 5 or 6 weeks and tracings of their tumors made. The results were recorded as the percentage of tumor growth regardless of size of the tumor. In a few experiments the mixtures were inoculated intraperitoneally and the time of appearance of ascites and day of death from tumor noted. In all instances the same tumor mixtures with normal allantoic fluid instead of NDV allantoic fluid were used as controls. For convenience of expressing the results of the experiments, failure of the growth of the tumor cell virus mixtures will be considered as the "adsorption" phase of the reaction and their subsequent growth as the "elution" phase. It is realized that the absolute proof that this is the explanation of the phenomenon must await further work.

1) *Effect of time and temperature on the growth of mixtures of NDV and Ehrlich carcinoma.* Fig. 1 represents the results of an average of 3 experiments. It can be seen that at all temperatures the inhibition of tumor growth took place immediately. The reaction was more complete and more sustained at both 4° and 37° than at 25°. There appeared to be some spontaneous elution at all temperatures but most marked at room temperature. The figures given for tumor growth at 5 hours may reflect a loss in viability of the tumor cells themselves as the normal allantoic fluid controls grew in only 90% of the instances at 25° and 37° in contrast to 100% for each of the other times and temperatures. The HA titers on the supernatant of the tumor cell virus mixtures which were done at the time of inoculation showed an initial drop immediately after mixing at all temperatures. This was followed by a gradual rise in titer so that after 3 to 5 hours the original titer was almost attained. As noted in the experiments with NDV and sarcoma 180 there was no absolute correlation between the rate of tumor growth and the HA titer. For example, although the HA titer for both mixtures at 4° and 37° had risen to almost the original value by 4 hours, tumor inhibition at these temperatures was still present.

TABLE I. Effect of Intraperitoneal Inoculation of Mixtures of NDV and 10% Ehrlich Carcinoma Held at 4° for 10 Min. 4 experiments.

NDV and Ehr.	NAF and Ehr.
3/10*	10/10
3/10	7/10
2/10	10/10
4/10	10/10

* Deaths from ascites over No. of mice inoc.

2) *Elution of NDV and Ehrlich tumor cell mixtures.* As mentioned above there was evidence of spontaneous elution (as measured by tumor growth), which was most prominent at 25°. When mixtures were kept at 4° for 10 minutes and then placed at 25°, elution also took place rapidly. In two different experiments no elution could be demonstrated when the mixtures were placed at 37° following adsorption at 4°.

3) *Effect of inoculating mixtures of tumor cells and NDV intraperitoneally.* When mixtures of equal parts of NDV and 10% Ehrlich carcinoma cells were held at 4° for 10 minutes and then inoculated intraperitoneally into mice, only a small per cent of those animals receiving the mixtures developed ascites, and it appeared long after the control animals had died. The results of these experiments are given in Table I.

In vivo experiments. It is apparent that inhibition of tumor growth took place when mixtures of Ehrlich carcinoma cells and NDV were inoculated after contact *in vitro*. We were therefore interested to see if any adverse effect could be demonstrated when the virus and tumor were inoculated separately or if the virus could be used as a therapeutic agent after ascites developed. Groups of 10 weighed mice were inoculated intraperitoneally with 0.2 cc of a 5% Ehrlich carcinoma suspension. They were then inoculated intraperitoneally with either NDV infected allantoic fluid or with normal allantoic fluid under the con-

TABLE II. Effect of Inoculating NDV Followed by Inoculation of Ehrlich Carcinoma Intraperitoneally, 4 Experiments.

Conditions of exp.	NDV	NAF	Avg day of death NDV	Avg day of death NAF
NDV				
1 cc & Ehr. immed.	6/10*	10/10	32.3	20.4
.2 cc " "	6/10	10/10	25.6	17
.2 cc " 10 min. later	8/10	10/10	33.4	15.5
.2 cc " 1 hr "	9/10	10/10	22.3	16

* Deaths from ascites over No. inoculated.

ditions specified in the experiments. Ten animals which had received virus only were included in each experiment. The mice were weighed individually at twice weekly intervals, to determine the time of development of the ascites. The day of death from ascites was also recorded. The results were judged on 1) the number of survivals 2) the average survival time and 3) the rate of development of the ascites.

Table II gives the results of the one category of experiments in which the virus in the form of infected allantoic fluid was given first. There were some survivors in all treated groups, the greatest number when virus and tumor cell suspension were inoculated almost simultaneously. The animals which received the virus developed ascites at a slower rate and survived longer than the controls. When the 0.2 cc of a 5% tumor cell suspension was given first and the same quantity of virus given immediately thereafter, there were no survivors although the NDV treated animals survived twice as long as the controls. When inoculation of virus was delayed for as much as 10 minutes after the tumor, no difference could be noted between virus and normal allantoic fluid treated animals. In other experiments daily inoculations of 0.2 cc of NDV failed to effect the development of ascites or failed to have an effect once the ascites had been allowed to develop.

Failure of growth of NDV in Ehrlich tumor. When 0.1 cc of NDV was inoculated directly into Ehrlich tumor of 1.0 cm in diameter and mice killed at daily intervals, the virus titer showed a gradual decrease. Table III. The virus did not affect tumor growth. One attempt to propagate NDV in Maitland type tissue culture with the Ehrlich tumor as the tissue source gave no evidence of survival of virus after 3 passages although chick embryo control flasks showed good growth. In addition 7 mice with well developed ascites were inoculated with NDV (0.5 cc). Daily titrations of the ascitic fluid showed no increase in the amount of virus, Table III. Numerous attempts to keep the virus in passage at 3-day intervals in mice with ascitic fluid resulted in the loss of virus.

TABLE III.
Persistence of NDV in Ehrlich Carcinoma.

Days after inoc.	Solid tumor titer*	Ascitic fluid titer*
Orig.	9	9
Immed.	6.5	5.6
1	6	5
2	4	5
3	4	3
6	0	1.75
7	0	0

* Expressed as the reciprocal of the LD_{50}.

Discussion. The results of mixing NDV and Ehrlich carcinoma cell suspensions *in vitro* and *in vivo* have been reported. It has been seen that when the two are mixed in the test tube inhibition of tumor growth takes place immediately. Further inoculation of the mixtures at room temperature for one to 3 hours has resulted in growth of these mixtures. These results are entirely comparable to those obtained with the sarcoma 180 cell suspensions and NDV.

In contrast, when tumor suspension and virus were mixed in the animal in the same proportions employed in the *in vitro* experiments only a very slight inhibition of tumor growth could be demonstrated and then only when virus and tumor were inoculated simultaneously. Since it was shown that virus and tumor mixed *in vitro* at 37° show impaired growth on inoculation, we are forced to seek other explanations for its failure to occur in

the animal. It may be that the virus is so rapidly changed in the animal body that it has no chance to react with the tumor cell, or the known inhibitors present in all animal tissues may interfere. Perhaps much more virus is needed to produce the effect *in vivo* than *in vitro*.

The results reported are in complete agreement with those reported by Kausche, Landschutz and Sauthoff, who had observed the same phenomenon with the PR8 strain of influenza and Ehrlich tumor cell suspensions. Their explanation of the phenomenon, *i.e.* growth of the virus in the tumor cells, are at variance with ours since we have been unable to demonstrate any increase in viral titer in either the ascitic fluid or in the solid tumor following direct inoculations. It appears to us that the tumor inhibiting effect is more likely to be due to an adsorption of virus on the cell surface which for some unexplained reason is capable of retarding tumor growth when these mixtures are inoculated either intraperitoneally or subcutaneously into animals. The fact that these same mixtures on further incubation or on a change of temperature from 4° to 25° are capable of growing when inoculated, seems to indicate a surface phenomenon.

Summary. 1. When NDV and Ehrlich were mixed at 4°, 25° and 37° and inoculated immediately, a much smaller percentage of tumors grew than in the NAF control mixtures. When the same mixtures were held at 25° or transferred from 4° to 25° for one to 3 hours, inoculation resulted in tumor growth. 2. When virus and tumor were mixed *in vivo* no tumor inhibitory effect was noted unless the virus was given intraperitoneally first and the tumor suspensions by the same route immediately thereafter. The course of development of ascites in mice was not affected by daily inoculations of NDV. 3. No evidence could be found for multiplication of the virus in the tumor cells either in the animal or tissue culture.

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Dextran as a Source of Liver Glycogen and Blood Reducing Substance.* (19922)

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(With the technical assistance of Jean Rogers and Mary Z. Schumpert.)

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In earlier experiments in this laboratory it has been observed that dextran, fed to human subjects, is not recovered in the stools. Engstrom and Aberg(1) have suggested that some of the dextran administered intravenously might be excreted into the gastrointestinal tract where it could be destroyed by bacterial action. Our own observations on dextran incubated with stools suggested that dextran disappears only very slowly, but Hehre(2) has since shown that there are large numbers of anaerobic bacteria present in the intestinal flora, and that it is these, rather than the aerobes, that are capable of splitting dextran. Although ingested dextran may be in part degraded and consumed by the intestinal bacteria, it still seemed to us of interest to learn more about the disappearance of dextran fed by mouth. The following experiments show that dextran feeding leads to a sustained increase in blood sugar and a rise in liver glycogen in the fasted rat, and brings about an

* This investigation was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

TABLE I. Blood Reducing Substance Following Administration of 5 ml of 18% Dextran by Stomach Tube to 24-Hr Fasted Rats.

Time in hr	0	1	2	3	4
No. of rats	6	4	4	2	2
Reducing substance (mg %)	80 ± 7.9	113 ± 3.5	100 ± 8.2	96.5 ± 2	95.5 ± 2

increase in the blood sugar of human subjects.

Experimental. Male albino rats of the Sprague-Dawley strain, weighing 250-350 g were fasted for 24 hours. A group of 10 control animals were killed and both fractions of liver glycogen were determined by the method of Bloom, Lewis and Schumpert(3). Glycogen was determined (as glucose equivalent) by means of the anthrone reaction. The fasted experimental animals were given 5 ml of 18% dextran in 0.9% saline by stomach tube. An initial blood sample was taken from the cut end of the tail and additional samples were taken at intervals after feeding. Copper tungstate filtrates were prepared and reducing substances were determined by the method of Nelson(4). In a series of four rats, both total and fermentable reducing substances were determined. Two or four hours after feeding, the rats were anesthetized with Nembutal and the liver was removed and immediately frozen and powdered. Trichloroacetic acid-extractable and total glycogen was determined on aliquots of the well-mixed liver powder. In order to determine whether glycogen or dextran might be present, other samples were allowed to stand at room temperature for 1.5 to 2 hours in order that glycogenolysis might occur. Dextran was added to parallel samples in order to determine whether dextranolysis could occur in the liver preparation.

To two human subjects, fasted for 12 hours, 100 ml of 20% dextran were given orally and blood sugar was determined at 0, 0.5, 1 and 2 hours thereafter. Two other patients were fasted 12 hours, 200 ml of 20% was given orally and blood sugars determined at the described intervals.

Results. The data of Table I show that the blood reducing substance in dextran-fed rats increases significantly in one hour after feeding and is still above the initial level after four hours. In Table II it is seen that the increase in blood reducing substance (of about the same extent but of shorter duration after feeding half the amount of dextran given to the first series of animals) is entirely fermentable. Table III presents the data on two human subjects given 20 g of dextran by mouth. The increases in blood sugar in 0.5 to 1 hour are of the same order as those seen in the rats.

In Table IV it is seen that there is a considerable increase in liver glycogen four hours after feeding dextran to previously fasted rats, and that allowing the liver samples to stand

TABLE II. Total and Non-Fermentable Blood Reducing Substance in 24-Hr Fasted Rats Given 5 ml of 9% Dextran by Stomach Tube.

Wt, g	Time after dextran	Blood reducing substance (mg %) Total	Non-fermentable
285	Control	99.2	0
	30 min.	116.2	0
	2 hr	86.1	0
	4 "	73.2	0
300	Control	80.1	0
	30 min.	121.7	0
	2 hr	74.8	0
	4 "	63.6	.25
265	Control	87.4	0
	30 min.	104.6	0
	2 hr	81.8	2.5
	4 "	67.7	.5
250	Control	65.5	12.7
	30 min.	97.6	0
	2 hr	81.8	4.3
	4 "	68.5	1.3

TABLE III. Blood Reducing Substance Following Administration of 100 ml of 20% Dextran to 12-Hr Fasted Human Subjects. (Values for reducing substance in mg %.)

	Subject I	II	III	VI
Control	83	100	90.5	118
½ hr	107	110	103	161
1 "	99	126	101	137
2 "	89	112	104	128

TABLE IV. Liver Glycogen Concentration in Fasting and Dextran Fed Rats.

	Alkali soluble, mg/100 g	TAA soluble, mg/100 g
10 fasting rats	130 ± 13*	31 ± 8
14 fasted rats 4 hr after dextran feeding		
Immediate analysis	751 ± 106	469 ± 66
Aliquot at room temp. 2 hr	71 ± 13	10 ± 2

* Stand. error.

TABLE V. Recovery of Added Dextran from Livers Undergoing Glycogenolysis.

No. of livers analyzed	Dextran added, mg/g	Dextran recovered, mg/g	
		Alkali soluble	TAA soluble
6	644	517 ± 31*	610 ± 8
8	736	720 ± 22	683 ± 20

* Stand. error.

The dextran was added to each of two samples, one of which was digested with alkali, the other of which was extracted with trichloroacetic acid. Time of incubation at room temp. was 2 hr.

for 2 hours at room temperature results in the disappearance of most of the glycogen. Dextran added to aliquots of the liver tissue and determined either as alkali-soluble or trichloroacetic acid-soluble anthrone-reacting substance, is recovered without substantial loss, so that no significant dextranolysis seems to occur under these conditions in liver tissue (Table V).

Discussion. The data show that in both rat and man the oral administration of dextran leads to a significant and sustained increase in blood reducing substance, most of which is fermentable. In the rat, this increase in blood sugar is accompanied by a significant increase in liver glycogen 4 hours after feeding. Dextran is therefore capable of being broken down in the intestine to products which yield glucose and glycogen in the animal. The early increase in blood sugar in both rat and man indicates that the intestinal breakdown of dextran may be a relatively rapid process, and it suggests that this may not be ascribable merely to bacterial action but more probably to an action of an enzyme or enzymes of the intestinal tract. Experiments now under way in this laboratory indicate that the latter possibility may be realized: suspensions of rat duodenal mucosa have been found to liberate glucose from dextran at rates which can account satisfactorily for the increases in blood sugar seen after feeding.

The authors wish to express their appreciation to Dr. Conrad Kinard who helped with the clinical study.

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Plasma Volume Determination in the Hypovolemic State Produced by Intraperitoneal Injection of a Non-Electrolyte Solution. (19923)

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It was noted in previous work(1), that following the subcutaneous injection of non-electrolyte containing fluids there were certain discrepancies in plasma volumes as determined by the RIHSA* method. These were manifested by failure to obtain reduction in plasma volume commensurate with the rise in hematocrit, and clinical signs of hypotension and dehydration. One of the possible factors considered was an abnormal rate of disappearance of the radioactive albumin in this pathophysiologic state. It was thought to be worth-

* Radioactive Iodinated Human Serum Albumin.

while to investigate the disappearance of radioactive iodinated protein under controlled conditions in the dog when a non-electrolyte carbohydrate solution was given intraperitoneally.

Methods. Six mongrel dogs weighing 18 to 20 kg on the standard kennel diet were studied. Control plasma volumes were obtained utilizing the radioactive iodinated albumin method previously described(2). The radioactive I^{131} protein was prepared from dog plasma according to the method of Fine and Seligman(3). In unanesthetized animals a polyvinyl catheter was introduced into the external jugular vein through a No. 13 needle and taped in position. A short No. 18 needle was wedged into the lumen of the catheter for convenience in sampling. Twenty cc of the radioactive plasma were injected into the opposite external jugular vein and periodic samples were withdrawn from the catheter for 30 minutes. The blood samples were placed in heparinized tubes and centrifuged at 2500 rpm for 30 minutes. The radioactivity of the supernatant plasma was determined and expressed as counts per minute. Plasma volumes were calculated from the 10 minute sample(2). After 30 minutes a 10% invert sugar solution,† 25 ml/kg, was introduced into the peritoneal cavity through a No. 18 needle. This infusion required 10 to 15 minutes. At 1, 2, 3, and 4 hours after the intraperitoneal injection simultaneous blood and peritoneal fluid samples were obtained. The radioactivity of one cc aliquot of these samples was determined as described above. In addition, the following chemical determinations were made on the samples: sodium and potassium concentration using a Barclay flame photometer and chloride concentration by the method of Schales and Schales(4). Four hours after the intraperitoneal infusion, 20 cc of radioactive plasma were again injected into the external jugular vein after obtaining a control blood sample, and periodic blood samples were collected from the polyvinyl catheter for 30 minutes.

† The 10% invert sugar in distilled water (Travert) employed in this study was kindly supplied by Dr. Robert P. Herwick, Medical Director of the Baxter Laboratories, Morton Grove, Ill.

TABLE I. Changes in Plasma Volume and Hematocrit 4 Hours Following Intraperitoneal Infusion of 10% Invert Sugar.

Dog		Plasma vol	Hematocrit
3	Control	1200	45.2
	4 hr PI*	1088	53.4
4	Control	1102	34.9
	4 hr PI	925	42.5
5	Control	908	49.8
	4 hr PI	655	55.6
6	Control	772	49.2
	4 hr PI	672	52.9
8	Control	903	41.9
	4 hr PI	975	47.7
9	Control	849	50.1
	4 hr PI	725	54.8

* PI = Post infusion.

TABLE II. Changes in Electrolyte Concentrations in Peritoneal Fluid Following Intraperitoneal Infusion of 10% Invert Sugar.

Dog No.	Time, hr	Electrolytes in meq/l Na	K	Cl
P–5	1			73.1
	2			91.6
	3			95.2
	4			97.4
	4½			102.2
–6	1	79.6		
	2			
	3	117.8	3	80.8
	4	120	3.18	103
–8	1	60.8	1.5	
	2	103.7	2.5	
	3	109.6	2.8	
	4	116	2.9	
–9	1	65.2	1.95	
	2	94	2.76	
	3	105	3.04	
	4	110	3.21	

Results and discussion. Table I summarizes the change in plasma volume 4 hours after the intraperitoneal injection. The average decrease in plasma volume was 145.5 cc or 16.7%. The average rise in the hematocrit was 15.3%. It may be noted that a decrease in plasma volume occurred in all but one animal. There was a uniform rise in the hematocrit determinations. The changes in plasma volume and hematocrit following intraperitoneal injection of the non-electrolyte solution indicates a significant shift of fluid from the intravascular compartment. This is in accord with the results of other investigators (5) who have demonstrated this fluid shift.

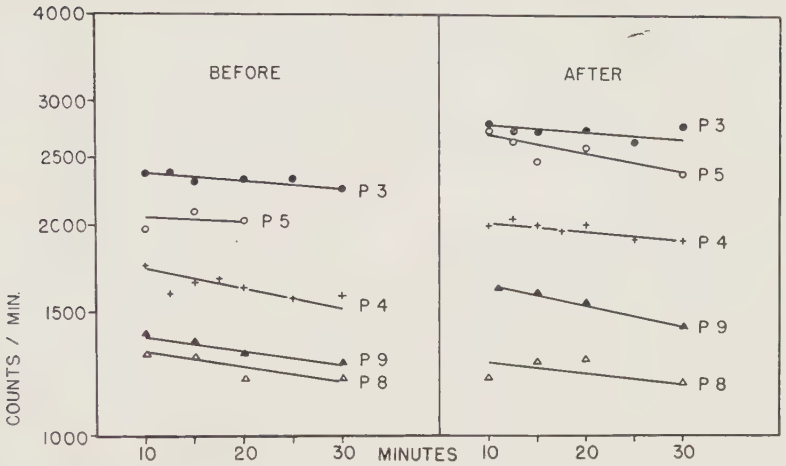

FIG. 1. One half hour disappearance curve of radioactive iodinated protein before and after peritoneal inj. of Travert.

It has been previously demonstrated that there is a transfer of electrolytes into peritoneal fluid when a non-electrolyte solution is injected into the peritoneal cavity(5). Table II indicates the electrolyte concentrations in the peritoneal fluid at various times following the injection.

Fig. 1 is a comparison of the ½ hour disappearance curves for 5 dogs before and 4 hours after the intraperitoneal injection. The slopes show little difference before and after the injection. The radioactivity of peritoneal fluid at intervals up to 4 hours after the injection averaged 10 to 33 counts per minute. These values are insignificant when compared to the activity of the plasma at the same time intervals. It would be logical to conclude that under the conditions of this experiment: 1. There is no significant change in the rate of disappearance of radioactive iodinated albumin from the blood stream in 30 minutes when a nonelectrolyte fluid (10% Travert) is injected into the peritoneal cavity in sufficient amounts to cause a significant fluid shift from the intravascular compartment. 2. Under these conditions intravenous radioactive protein did not enter the peritoneal fluid in significant amounts over a 4-hour period.

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Oxytocic Activity of Purified Vasopressin. (19924)

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In the fractionation of aqueous extracts of posterior pituitary glands, Kamm and coworkers(1) obtained preparations high in oxytocic activity, with small amounts of pressor and antidiuretic activities. They also obtained preparations high in pressor and antidiuretic activities with low oxytocic activity. Relatively, however, oxytocin preparations were freer of pressor activity than *vice versa*. Since their most potent pressor fractions still possessed considerable oxytocic activity, they suggested that the pressor hormone might possess inherent oxytocic activity. Following the appearance of the work of Kamm and coworkers, several alternative procedures for the separation and purification of the active principles from the posterior pituitary have been employed. These procedures have been reviewed extensively(2). None of the methods has yielded a pressor preparation completely devoid of oxytocic activity. However, on these preparations there was no analytical data which could exclude the possibility of contamination with oxytocin.

With the more refined technics recently available—countercurrent distribution(3) for purification and starch column chromatography(4) for analysis of the constituent amino acids—it has been possible to prepare and to analyze high potency oxytocic material(5,6) and high potency vasopressor material(7). The present paper reports the pharmacological testing of such preparations for oxytocic, pressor, and antidiuretic activities.

Experimental. The vasopressin preparation used in this study was prepared from desiccated beef posterior pituitary lobes by the procedure of Turner, Pierce, and du Vigneaud(7), except that a greater number of transfers was used in the countercurrent distribution procedure and in the later stages the distribution was carried out at 10°. Analysis of a hydrolysate of this material by the starch column chromatographic technic of Moore and Stein(4) showed the presence of the 8 constituent amino acids of high potency beef vasopressin preparations(7)—phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, arginine, and cystine—in molar ratios approximating 1:1, plus 3 moles of ammonia per mole of any one amino acid. No other amino acids could be detected. Since the presence of phenylalanine in vasopressin preparations has been questioned and the presence of isoleucine has been suggested (8), we would add that microbiological assay of a hydrolysate of a vasopressin preparation for L-phenylalanine, performed in the Cornell Laboratories by Dr. Dorothy S. Genghof using *L. arabinosus*(9), gave results in good agreement with the starch column analysis. Furthermore, when isoleucine was added to a hydrolysate of vasopressin prior to starch column analysis a new peak appeared at the isoleucine position which was clearly distinguishable from the phenylalanine peak.

Pressor assays were made on the preparation by comparing its effect with that of U. S. P. standard posterior pituitary powder on the arterial blood pressure of cats under phenobarbital anesthesia. Operative and mechanical procedures were similar to those described by Kamm and coworkers(1) for assays using dogs. The preparation consistently showed 600-650 pressor units per mg by this method. Assayed for oxytocic activity by the chicken blood pressure method of Coon(10), the preparation appeared to possess an activity of 80-90 oxytocic units per mg. Oxytocic

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‡ A portion of the experiments was completed in the National Institute for Medical Research, Mill Hill, London, through the kind hospitality of Sir Charles Harington and Dr. W. Feldberg.

assays were then performed on the rat uterus immersed in de Jalon solution(11) at 32.5°. An oxytocic activity of approximately 30 units per mg was found for this preparation. This relationship of 1 oxytocic unit to each 20 vasopressor units even held after a considerable loss of activity had been encountered, when some of the preparation was dissolved in 0.25% acetic acid and distributed in ampoules which were sealed and sterilized. The activity of the contents in this instance happened to fall from 20 to 10 vasopressor units per ml. Coincidentaly the oxytocic potency for the rat uterus fell from 1 unit per ml to 0.44 unit per ml.

An antidiuretic assay on the 600-650 unit per mg vasopressin preparation, using rats and diabetes insipidus dogs, also gave 600-650 antidiuretic units per mg, in reference to the U. S. P. standard posterior pituitary powder. Another preparation showing 450 units per mg in the cat pressor assay also showed 450 units per mg in the antidiuretic assays.§

A purified preparation of oxytocin(6,13) assaying approximately 500 oxytocic units per mg by the chicken blood pressure method and showing no detectable pressor activity in the cat, showed an antidiuretic activity of less than 0.5 unit per mg in the diabetes insipidus dog.

Discussion. Of the 8 amino acids present as constituents of high potency vasopressin preparations, 6 are also found in hydrolysates of highly purified oxytocin preparations(6), that is tyrosine, proline, glutamic acid, aspartic acid, glycine, and cystine. The remaining 2, arginine and phenylalanine, are absent in oxytocin preparations while leucine and isoleucine are present. Since there is good reason to believe that these oxytocin preparations are pure or nearly pure samples of the oxytocic principle, the leucine/isoleucine content of vasopressin preparations is on this basis a measure of the maximum contamination by oxytocin. In the analysis of the 2 mg sample of vasopressin by starch column chromatography, it would be possible to detect 0.7% contamination by oxytocin by the appearance of a leucine/isoleucine peak in the chromatogram. Since the vasopressin preparation used in the present study contained no detectable leucine or isoleucine, the contamination by oxytocin (500 units per mg) must be less than 0.7%, or less than 3.5 oxytocic units per mg. From the oxytocic assay of 30 units per mg, one is led to conclude that vasopressin has intrinsic oxytocic activity as one of its biological properties, unless there is another oxytocic contaminant, which at the moment we have no reason to suspect. The finding that with decline of vasopressor activity in a dilute solution of vasopressin there was a corresponding and proportional loss of oxytocic activity supports the conclusion made.

The fact that the most highly purified vasopressin preparation shows the same number of units per mg in the antidiuretic assay as in the pressor assay offers convincing evidence that the antidiuretic and pressor activities are truly due to the same chemical compound, which has long been suspected. The fact that highly purified oxytocin preparations show no pressor activity by the blood pressure method and only a slight trace of activity in the extremely sensitive antidiuretic assay leaves no doubt that oxytocin does not possess pressor or antidiuretic activity.

It should be recalled that Coon(10) found that when pressor activity strongly predominated in various preparations, exaggerated responses were obtained by the chicken blood pressure method for oxytocic activity in reference to the uterine strip method. For example, he reports, for a solution possessing 1.5 units per cc by the uterine method and an oxytocic/pressor ratio of 0.06, that the chicken depressor method gave 5 units per cc, 3.5 times the uterine assay. This observation compares well with the results of the present study on high potency vasopressin. Here the oxytocic/pressor ratio is 0.05 and the chicken depressor assay is 2.7-3.0 times the uterine assay.

The recent experiments with lactating sows

§ Preliminary assays of antidiuretic potency using rats according to Burn(12), carried out with the capable assistance of Mrs. Jacqueline E. Parton, had indicated that a vasopressin preparation of about 400 pressor units per mg possessed approximately 400 antidiuretic units per mg in reference to the U. S. P. standard powder.

reported by Whittlestone(14) on milk-ejecting activity of high potency preparations of both oxytocin and vasopressin, prepared in the Cornell Laboratories, are of interest in connection with the question of the inherent oxytocic activity of vasopressin. He found that an oxytocic preparation of approximately 500 units per mg(6,13) showed a milk-ejecting activity equivalent to its oxytocic activity and that a vasopressin preparation of 400 pressor units per mg(7) showed a milk-ejecting activity equivalent to that which would be expected of approximately 80 units of oxytocin. Cross and van Dyke(15) have made similar comparisons in lactating rabbits by the method of Cross and Harris(16), using an oxytocin preparation with a potency of about 500 units per mg, and the 600 unit per mg vasopressin preparation of the present study, both of which had been prepared in the Cornell Laboratory. The results of Cross and van Dyke were in good agreement with those reported by Whittlestone.

Summary. Evidence has been obtained with a highly purified preparation that vasopressin, in addition to its pressor and antidiuretic activities, possesses intrinsic oxytocic action as one of its pharmacological properties, in contrast with purified oxytocin which appears not to possess pressor or antidiuretic activity. For each 100 units of vasopressor-antidiuretic activity there is intrinsic "oxytocic" activity represented by approximately 5 units as determined by the isolated rat uterus assay or by approximately 13-15 units by the chicken blood pressure depressor assay. The evidence that vasopressin possesses milk-ejection activity about one-fifth that of oxytocin has been discussed.

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Assay of Botulinum a Toxin with Goldfish.* (19925)

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The purpose of this report is to describe the successful use of the common goldfish as an assay animal for botulinum toxin. Goldfish have the obvious advantage that they are inexpensive and can be maintained healthy in a very small space.

The goldfish has been used extensively as a laboratory animal in toxicity tests. Pittenger and Vanderkleed(1), Pittenger(2), and Lapenta(3) observed specific reactions in

* Publication of the Department of Biophysics.

goldfish when treated with digitalis and with toxins. They obtained highly reproducible results and advocated the use of goldfish in the standardization of doses of these agents. Stevenson, Helson, and Reed(4) showed that goldfish can be poisoned with botulinum toxin. On the basis of these findings and in view of the need for an animal less expensive and easier to handle than the mouse, it was decided to try to develop an assay technic for botulinum toxin using the common goldfish.

Materials and methods. A preparation of botulinum A toxin containing 6 x 10^6 mouse 50% lethal doses per ml was used in these experiments. The preparation was obtained through the courtesy of Dr. Eugene M. Sporn at Camp Detrick, Frederick, Md. It was stored at 4°C for about a year prior to use, but the mouse assay was kindly carried out by Dr. Sporn at the time the experiments on goldfish were under way. In most tests, 10-fold dilutions of the toxin sample were made with specially prepared .1 M phosphate buffer at pH 7 containing 0.2% gelatin. Common goldfish, *Carassius auratus,* from 2-3 in. in length and from 2-4 g in weight were used in these experiments. The diluted toxin solutions were introduced intraperitoneally. After an examination of the internal anatomy of the goldfish, it was decided that the least amount of damage to vital organs would be caused if the needle were inserted between the pelvic fins and directed toward the backbone. During the injection, the fish were held in a small net between the thumb and the forefinger of the left hand. Twenty-seven gauge, ¼ in. needles were used, and 0.05 ml of solution was injected into each fish. Usually 10 fish per dilution were used. After the inoculation, the fish were stored in 3 gallon glass aquaria, not aerated. The criterion chosen for deciding whether or not a fish was dead was the complete cessation of movements of the mouth and opercula. Deaths which occurred within 12 hours after injection were classified as non-specific. End points were calculated by an algebraic modification(5) of the Reed and Muench method(6). They were calculated on the basis of mortality ratios observed 36 hours after inoculation. Experiments were carried out to determine the effect of temperature on the 50% mortality end point in fish. To this end the fish were held in the aquaria at various temperatures after inoculation. Those fish held at 35° were first acclimatized to that temperature prior to the inoculation. This was done by raising the temperature of a thermostatic bath containing the fish approximately 1° per day.

TABLE I. Effect of Toxin Concentration on Mortality Ratios.

Dilution	Mortality ratio
Uninj. control	0/10
Buffer control	0/10
10^{-2}	10/10
10^{-3}	9/10
10^{-4}	2/10
10^{-5}	0/10

TABLE II. End Points Calculated from Independent Assays on Same Preparation of Toxin.

3.8
4
3.8
3.5
4.1
3.5
3.6

Experimental results. A representative experiment showing the effect of toxin concentration on mortality ratios is shown in Table I. The end point of this particular experiment calculated by the method of Reed and Muench is 3.6. Reproducibilities of the end points were determined by carrying out seven independent titrations on the same toxin preparation. The results are shown in Table II. The mean end point is 3.8 and the standard deviation of the values is 0.24. Since these end points are expressed as logs of concentrations, this result indicates that end points are reproducible within about a 2-fold concentration of toxin.

Experiments were carried out using 4-fold and 2-fold dilutions to determine whether there was any effect of dilution interval on the end point calculation. Values of 4.1 and 3.5 were obtained respectively. It was observed that mortality ratios varied from zero to 1 over a concentration range of between 10 to 100 fold. On the basis of this result, it was decided that no substantial advantage would ensue from the use of dilution intervals less than 10-fold.

Burgen *et al.*,(7) observed that tempera-

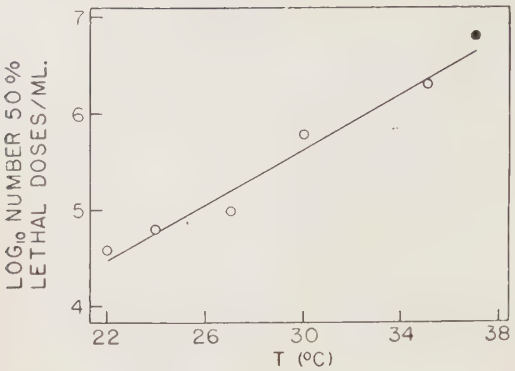

FIG. 1. Variation of the 50% lethal dose of botulinum A toxin in goldfish with temperature. Open circles–goldfish assays; closed circle–mouse assay.

ture variation affected the response of rat phrenic nerve-diaphragm preparations to botulinum A toxin. In view of this result, the effect of temperature on the 50% mortality end point of botulinum A toxin in goldfish was investigated. It was found that the end point values were increased and that the fish at higher temperatures died earlier than those receiving the same dose but kept at lower temperatures. The data obtained are plotted in Fig. 1. They show that the logarithm of the titer expressed as 50% lethal doses per ml increases with the temperature in essentially a linear manner. The straight line was fitted to the data by the method of least squares. When this line is extrapolated to 37°, a value is obtained which is in excellent agreement with the assay value for the toxin preparation in mice.

It should be pointed out that the apparently excellent agreement between the extrapolated value for goldfish at 37° and the value for mice may be in part fortuitous. These end points are determined as 50% lethal doses per unit volume of toxin solution. There is about a five-fold difference in body weight between the mouse and the goldfish in this experiment. On this basis, the susceptibility per unit body weight would be higher for the mouse than for the fish. Furthermore, the mouse end point was calculated from mortality ratios at the end of 48 hours and the fish end points were calculated from mortality ratios observed after 36 hours. Thus, the experiments in fish and in mice are not absolutely comparable. Nevertheless, it is apparent that a large fraction of the difference between the end point observed with fish at room temperature and the end point observed with mice can be attributed to the effect of temperature.

The effect of temperature on the mortality dose of botulinum A toxin can be interpreted in terms of conventional chemical kinetics. If the natural logarithm of the number of 50% mortality doses per ml is plotted against the reciprocal of the absolute temperature, a straight line can be fitted. From the slope of this straight line, a heat of activation for the over-all killing mechanism can be evaluated as 60,000 calories per mole. At least one positive statement can be made on the basis of this figure. It is much too high to be consistent with the assumption that the temperature effect is the result solely of a mechanism involving translational or rotational motion of the toxin particles. The reason for this is that such motion depends primarily upon viscosity which has a heat of activation of only about 4,000 calories per mole.

This heat of activation could arise from any one of a number of different kinds of mechanism. It could be the heat of dissociation of toxin aggregates or of toxin inhibitor complexes. It could be the heat of activation of a direct reaction between the toxin and some center for nerve impulse transmission. It could be the difference between the heat of association of some substance essential in nerve metabolism with its substrate and of the same substance with toxin behaving as an inhibitor. There is little evidence available to permit a selection among these and other possible alternatives. The idea that the heat of activation may be the heat of dissociation of essentially non-effective aggregates into effective smaller toxin units gains some support from the known dissociability of the toxin material at pH values above 7(8).

Summary. A method has been established for the assay of botulinum A toxin in goldfish. When 10 fish are inoculated per dilution and the dilution interval is 10-fold, the end point distribution was found to have a standard deviation of about 0.24 log unit. The end points are strongly temperature dependent; they decrease about a hundred-fold for a temperature decrease of 15°C. The end point

in fish at 37° has approximately the same value as that in mice.

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Blood Volume Studies in Pernicious Anemia and Non-Tropical Sprue. (19926)

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It is well recognized that patients with pernicious anemia in relapse tend to retain fluids. Clinically, this may be manifested by minimal edema of skin and subcutaneous tissues or by frank dependent edema. Clinical evidence of increased water retention may be evident during the early phase of effective therapy as shown by increase of peripheral edema and occasionally by the appearance of pulmonary edema. Meulengracht(1) demonstrated that persons in relapse showed decreased diuresis following ingestion of large quantities of water. Vaughan(2) reported retention of fluid in 12 patients with pernicious anemia in relapse from the onset of treatment to the peak of reticulocytosis after which there was a period of diuresis and return to normal fluid balance. Gibson(3) reviewing the work of many investigators, noted no general agreement in reports of plasma volume changes in patients with pernicious anemia. Using Evans blue dye (T-1824) he observed a fall in plasma volume and rise in red blood cell and total blood volume after treatment. He made no studies during the period of reticulocytosis. These variable results plus the frequently observed discrepancy of a sharply elevated reticulocyte peak after specific anti-pernicious anemia therapy, with no increase in hemoglobin or red blood cell values, prompted the following investigation. Hemodilution resulting from increased plasma volume could explain this seeming anomaly.

Methods. This study included one patient with non-tropical sprue and four persons with typical Addisonian pernicious anemia in relapse. Each case in the latter group showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, and megaloblastic bone marrow. Roentgen examinations of gastrointestinal tract, urinalyses and blood chemistry determinations disclosed no abnormalities. The patients were treated with either intramuscular injections of liver extract, vit. B_{12} or citrovorum factor. The subject with non-tropical sprue showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, megaloblastic bone marrow and flat glucose tolerance curve. There were frequent liquid stools containing abundant fatty acid crystals. Roentgen evidence of spasm, segmentation and "puddling" of the barium column in the small intestines was reported. He was treated with citrovorum factor intravenously. The results of this study will be presented elsewhere(4). In all patients hemoglobin and red blood cell determinations were made 3 times a week, leukocyte counts once a week, and reticulocyte estimations daily. Blood volume determinations were made twice weekly using P^{32} labeled erythrocytes(5). Red cell mass was calculated from the total blood volume and hemato-

TABLE I. Hematologic Data, Red Blood Cell and Total Blood Volumes in a Patient with Non-Tropical Sprue (N.) and 4 Patients with Pernicious Anemia in Relapse Treated with Liver Extract, Citrovorum Factor and Vit. B_{12}.

Patient	R.B.C. $\times 10^6$	Retic., %	R.B.C.V., ml	T.B.V., ml	Day of treatment
N.	1.67	.5	810	3540	1
	1.55	2.6	800	3420	4
	1.92	14.4	895	3140	7
	2.62	4.2	990	2980	11
W.	1.85	.8	805	3610	1
	1.58	1.8	655	3120	4
	1.95	16.6	860	3490	7
	2.42	5.1	1010	3430	11
C.	1.30	1.1	355	2200	1
	1.15	2.1	346	2430	4
	1.60	17.8	460	2200	7
	1.67	21.4	475	2150	9
	2.11	7.8	530	2060	12
S.	2.94	1.3	1485	3915	1
	2.95	2.1	1380	3770	6
	3.21	4.5	1490	3720	9
	3.55	3.3	1525	3575	11
	3.87	1.3	1730	4145	17
K.	1.82	.6	930	4650	1
	1.69	.4	915	4620	4
	2.16	18.6	1080	4890	8
	2.97	8.3	1170	4110	12
	3.19	2.2	1430	4770	16

R.B.C.V. = Red blood cell vol. T.B.V. = Total blood vol.

crit, correcting the latter by a factor of 0.95 (6).

Results. All of the patients showed satisfactory hematologic and clinical remissions. Reticulocyte responses were in the optimal ranges and regeneration of red blood cells took place rapidly. (Table I.) None of the subjects with pernicious anemia showed any clinical evidence of fluid retention during the period of observation. Increase in hemoglobin and red blood cells was noted at about the same time that significant reticulocytosis was observed so that the aforementioned discrepancy did not obtain in the cases observed. Blood volume changes were not significant. As a rule, the gain in red cell mass was offset by a loss of plasma volume so that total blood volume remained constant. Since none of these subjects showed any clinical evidence of edema and no lag in increase of red blood cells occurred during the period of reticulocytosis, no comment is possible as to alterations in fluid balance when either of these phenomena is observed. Further studies are indicated in such cases.

The patient with idiopathic steatorrhea had moderate pre-tibial edema before treatment. At the peak of reticulocyte response a moderate amount of subcutaneous fluid was eliminated. Plasma volume decreased more than the proportional increase of red cell mass so that total blood volume fell during this period. This decrease of blood volume despite an increase in red cell mass was more marked 5 days later, at which time subcutaneous pretibial edema was virtually gone. Whether edema observed during pretreatment period was due to cardiac dysfunction resulting from anemia of myocardium, increased capillary permeability, alterations in electrolyte balance or serum protein alterations cannot be determined from this single case. Further studies in sprue and related diseases are contemplated.

Summary and conclusions. 1. During the period of reticulocyte response, the blood volume in 4 patients with pernicious anemia remained constant, an increase in red cell mass being balanced by reduction of plasma volume. 2. One patient with idiopathic steatorrhea,

edema and macrocytic anemia showed diminution of total blood volume despite a rise in red cell mass during the period of reticulocytosis.

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Poliomyelitis. I. Propagation of the MEF1 Strain of Poliomyelitis Virus in the Suckling Hamster.* (19927)

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The work reported here was initiated originally with the thought of developing a high titered virus which could be used to produce a more practical complement-fixation antigen for the diagnosis of poliomyelitis. Since the Syrian golden hamster appears to be less prone to spontaneous virus infections than the Swiss white mouse it was chosen for the serial transmission of the MEF1 strain(1,2), Type 2 poliomyelitis virus. While our work was in progress Casals, Olitsky and Anslow(3,4) and Selzer, Sacks and van den Ende(5) adapted the MEF1 strain of poliomyelitis virus to newborn mice, but no modification in virulence of the virus for primates was reported. During the course of our work, however, it was conceived that continuous passage of the virus in young hamsters might result in the production of higher concentrations of virus than are ordinarily attained in mice and that through adaptation or fixation for the species, in the Pasteurian sense, the virus might broaden its host range and thereby lose virulence for those species ordinarily more susceptible. Could these hopes be realized without loss of antigenicity by the virus, material progress would be made in the development of a practical diagnostic antigen as well as immunizing agents in the form of inactivated or live virus vaccines. Adaptation to new hosts, especially the chick embryo, would further expand the possibilities of research and greatly simplify the preparation of diagnostic and prophylactic antigens.

The present report describes the development of a passage strain of MEF1 poliomyelitis virus carried in 7- to 10-day-old suckling hamsters and of another strain carried in 2- to 4-day-old hamsters.

Materials and methods. Virus strains. The MEF1 Lansing type (Type 2) strain of human poliomyelitis virus was obtained as a 50% glycerine-water mouse brain suspension through the kindness of Dr. Peter K. Olitsky and Dr. Jordi Casals of the Rockefeller Institute for Medical Research, New York, N. Y. The strain was passed by intracerebral inoculation for 9 passages in Swiss white mice and was thereafter carried intracerebrally in 6- to 8-week-old Syrian golden hamsters. A virus pool was prepared from the nervous tissues harvested from the 30th to the 34th hamster passages in the following manner: the brain and spinal cord tissues were aseptically removed from paralyzed hamsters and were thoroughly ground in a TenBroeck grinder; a 10% tissue suspension of the

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† Sodium chloride 9 g; potassium chloride .3 g; calcium chloride .2 g; sodium carbonate .2 g per liter of solution.

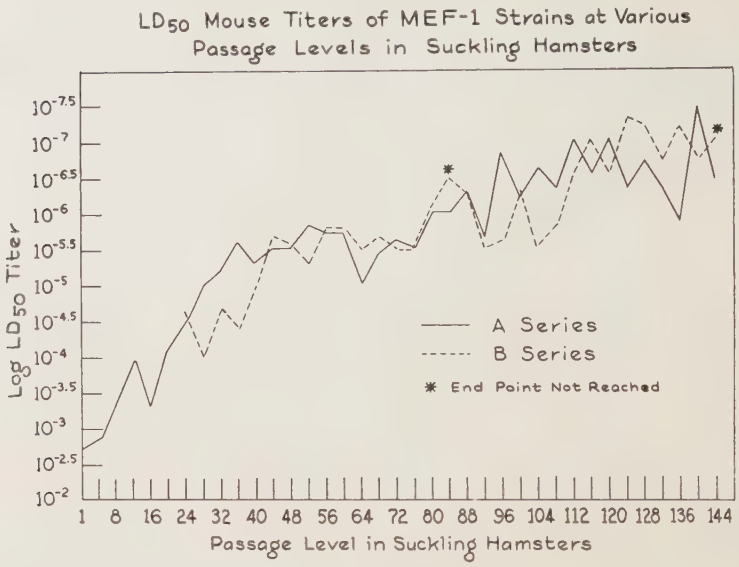

FIG. 1.

ground tissues was made in sterile Ringer's solution;† the suspension was centrifuged for 30 minutes at 2500 rpm in a size 1 International anglehead centrifuge; the supernate from this preparation was used to initiate the A series of passages in suckling hamsters. The same method of preparation was used in making tissue suspensions for subsequent passages in hamsters. A *virus pool* prepared from the 64th hamster passage of the MEF1-A strain described below was used in serum neutralization tests. The *Wallingford strain*(2), Type 2 poliomyelitis virus, shown in Table II, represented the first monkey passage of a virus suspension designated WLFD IV G981, which was received from Dr. Howard A. Howe, of the Poliomyelitis Research Laboratory, the Johns Hopkins University, Baltimore, Md. The *Brunhilde strain*(2), Type 1 poliomyelitis virus, represented the 3rd monkey passage of a virus suspension which was also obtained through the courtesy of Dr. Howe.

Passage method. A 0.03 ml dose of inoculum was injected intracerebrally in suckling hamsters in the A series passages. Passage in the 2- to 4-day-old hamsters of the B series was made with a 0.02 ml dose intracerebrally. In the later passages Ringer's solution was replaced by isotonic sodium chloride solution as a suspending medium. Brain and spinal cord tissues were harvested for passage. *Immune sera.* An anti-Lansing immune hamster serum was prepared by hyperimmunization of hamsters which had survived intramuscular injection of the MEF1 strain. A series of intramuscular injections starting with 0.25 ml of a 10^{-4} dilution of virus tissue suspension and ending with 0.5 ml of a 10^{-1} dilution was given at weekly intervals. An anti-Lansing immune monkey serum was prepared by hyperimmunization of a convalescent monkey which had been intracerebrally injected with the WW, Type 2 strain of poliomyelitis virus(6). The Armstrong strain(2) of Lansing type virus was used by the intramuscular route for hyperimmunization.

Neutralization tests. Undiluted hamster or monkey serum was mixed with an equal volume of a 20% virus tissue suspension or tenfold dilutions thereof, and the mixtures were incubated at room temperature for 90 minutes. Groups of 4 to 5 mice weighing 12 to 14 g were injected intracerebrally with 0.03 ml of the serum-virus mixtures. Daily observations were recorded for 21 days. Protective indices were calculated on the basis of the difference between the log LD_{50} titers of the virus in the presence of normal and immune serum. The neutralizing potency of the chimpanzee sera was calculated by the Reed and Muench method(7) on the basis of serial

TABLE I. Identification of the Two Hamster-Adapted MEF1 Strains of Poliomyelitis Virus. Serum neutralization tests.

Virus strain, passage series	Hamster passage	Serum	Serum dilution	Mortality ratio of mice inoc. intracer. with serum and dilutions of virus 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	LD_{50} titer of virus	Protective index
MEF1–A	83	Normal hamster	Undil.	5/5	5/5	5/5	2/5	0/5	0/5	$10^{-4.83}$	676
		Immune " *	Undil.	2/5	0/5	0/5	0/6	1/3		$10^{-2.00}$	
	130	Normal monkey	1:8		5/5	4/4	5/5	5/5	1/5	$10^{-6.63}$	426
		Immune " †	1:8		5/5	2/5	1/5	0/5	0/5	$10^{-4.00}$	
MEF1–B	126	Normal monkey	1:8		5/5	5/5	5/5	5/5	2/5	$10^{-6.83}$	1440
		Immune " †	1:8		4/5	2/5	0/5	0/5	0/5	$10^{-3.67}$	

* Lederle hyperimmune hamster serum.
† " " monkey " .

dilutions of serum tested against a predetermined number of LD_{50} doses of virus.

Experimental. Series A hamster passage. The A passage series was initiated on July 16, 1948, in 7- to 10-day-old suckling hamsters by intracerebral inoculation of hamster pool virus. Brain and spinal cord tissues were harvested at 2 to 4 days when the hamsters showed paralytic symptoms, usually in one of the forelegs. Passages were routinely made in one or more litters of suckling hamsters. The incubation period gradually shortened so that by the 121st passage symptoms began to appear as early as 24 hours after intracerebral inoculation. This young hamster passage sequence, designated the A series, has been maintained for 150 consecutive generations and is still in progress. The inoculum for each passage was titrated intracerebrally in tenfold dilutions in groups of 4 to 5 Swiss mice and the titers for every 4th passage, calculated by the method of Reed and Muench(7), are presented in the accompanying chart. There was a gradual increase in the concentration of virus demonstrated in the nervous tissues. From the 96th hamster passage onwards the titers rarely fell below $10^{-6.5}$ and were often found to be $10^{-7.0}$ or greater. In the 140th passage, for example, the titer reached $10^{-7.4}$.

Series B hamster passage. At the 23rd passage level in 7- to 10-day-old hamsters a subpassage series was started on February 23, 1949 in 2- to 4-day-old hamsters. This series was labeled "B" and the first such passage was designated B-24. The intracerebral dose was reduced to 0.02 ml in deference to the size of the baby hamsters but otherwise the passage technic was the same as that used in the A series of hamster passages. Excluding the 23 passages in 7- to 10-day-old hamsters, this substrain (series B) has been maintained for 116 consecutive passages in 2- to 4-day-old hamsters and is still in progress. At each passage level the inoculum was titrated in mice as in the A series and the LD_{50} titers for every 4th passage are presented in the accompanying chart. The titers attained in this series are equally as high as those demonstrated in the A series.

Identification of the passage virus. The details of the mouse neutralization tests are summarized in Table I. The 83rd hamster passage virus of the A series was tested against MEF1 immune hamster serum. Similarly, the 130th passage of the A series was tested against the Lederle anti-Lansing immune monkey serum. This same immune monkey serum was used against the 126th hamster passage virus of the B series. The data obtained in these three neutralization tests clearly indicate that the virus strains in the A and B passage series is Lansing type human poliomyelitis virus. The identity of the passage strains as poliomyelitis virus is further implied by the ability of the Wallingford strain, which is a Lansing type of human poliomyelitis virus, to elevate the neutralizing titer of the chimpanzee sera for the MEF1-A strain of virus (Table II).

Virulence tests in monkeys and chimpanzees. At the 54th passage level of the A strain of virus, 2 Rhesus monkeys were injected intramuscularly with 5 ml each of a 20% suspension of pooled hamster brain and cord tissue

TABLE II. Oral Administration of Poliomyelitis Virus to Chimpanzees. Neutralization tests at various intervals after feeding.

	Administration of poliomyelitis virus					Serum taken .... days later	Dilutions of serum neutralizing various LD_{50} doses of MEF1-A strain virus			
	Virus fed	LD_{50} titer in mice	Suspension, %	Vol., ml	Date fed			24 LD_{50}	18 LD_{50}	6 LD_{50}
Chimp. No. 1						0	Pre-feeding	<1:2	<1:2	<1:2
	B-82*	$>10^{-6.5}$	20	10	7/26/50	42	Post-1st feeding	—	1:16	1:238
	B-84	$>10^{-6.5}$	20	5	9/ 7	140	Post-2nd feeding	—	1:5	1:53
	B-94	$10^{-5.8}$	20	5	1/26/51	14	Post-3rd feeding	1:378	1:436	1:3540
	Brun.†	—	20	10	2/12	67	Post-Brun.	1:138	1:862	1:2380
	Wall.‡	—	10	7	4/23	29	Post-Wall.	—	1:654	1:3540
								30 LD_{50}	24 LD_{50}	10 LD_{50}
Chimp. No. 2						0	Pre-feeding	<1:2	<1:2	<1:2
	B-82*	$>10^{-6.5}$	20	10	7/26/50	42	Post-1st feeding	—	—	1:37
	B-84	$>10^{-6.5}$	20	5	9/ 7	140	Post-2nd feeding	1:3	—	1:76
	B-94	$10^{-5.8}$	20	5	1/26/51	14	Post-3rd feeding	1:4	1:158	1:111
	Brun.	—	20	10	2/12	67	Post-Brun.	1:5	1:662	1:258
	Wall.	—	10	7	4/23	29	Post-Wall.	1:14	—	1:798

* B-82 = 82nd passage in suckling hamsters, B series, MEF1 poliomyelitis virus.
† Brun. = Brunhilde, Type 1 poliomyelitis virus.
‡ Wall. = Wallingford, Type 2 " " .

which had a mouse LD_{50} titer of greater than $10^{-6.5}$. Both animals became paralyzed and died showing typical symptoms of poliomyelitis. At the 60th passage level of the B series of virus, 7 Rhesus monkeys were injected intracerebrally with 1 ml each of a 20% pooled hamster brain and cord tissue suspension having a mouse LD_{50} titer of $10^{-5.8}$. One of the 7 monkeys became paralyzed but survived, whereas the remaining 6 animals remained symptom-free. Similarly, 13 monkeys were injected intracerebrally with 1 ml each of a pooled hamster brain and cord tissue suspension of the 64th passage of the B series of virus, having an LD_{50} titer of $10^{-5.5}$ in mice. Two of the monkeys developed partial paralysis; two others became completely paralyzed but 9 remained symptom-free.

The apparently reduced virulence of the B series passage virus for Rhesus monkeys prompted a feeding test in chimpanzees. Accordingly, two chimpanzees were bled and then given feedings of 20% brain and cord suspensions representing the 82nd, 84th and 94th hamster passages of the B series of virus. The dates of feeding, volume and mouse LD_{50} titers of the suspensions fed are given in Table II, together with the serological findings. Serum samples were collected after each feeding of the B series virus and again following the feeding of Brunhilde and Wallingford virus suspensions. No virus was recovered from the stool samples collected after the feeding of the B series hamster passage virus, but Brunhilde virus was recovered by the inoculation of monkeys with stool samples collected from the chimpanzees 7, 10 and 14 days after being fed. Similarly, virus was recovered from stool samples collected 11 and 14 days after the feeding of Wallingford virus. The chimpanzees remained free of nervous symptoms throughout the course of the experiment.

The post-feeding serum samples, together with the samples obtained prior to feeding, were tested against the 64th hamster passage virus of the A series by mouse neutralization tests. The results, summarized in Table II, clearly indicate that the series B virus is capable of stimulating a systemic immune re-

sponse when administered by the gastrointestinal route, and that when given orally the strain is lacking in virulence for the chimpanzee. It is of further interest that the feeding of the Brunhilde and Wallingford strains of virus failed to induce symptoms in the treated animals and that antibody titers for the A series passage virus increased following the feeding of these heterologous strains. This observation takes on added interest and significance in the light of the recent reports of Casals *et al*(8) and Sabin (9) that Brunhilde type infection in man may be accompanied by the appearance of heterotypic Lansing type complement-fixing and neutralizing antibodies in the convalescent serum.

Infectivity by the intramuscular route in young mice. It was observed that both the A and the B series passage strains of MEF1 hamster-adapted virus would induce paralysis and death in 12 to 14 g white Swiss mice, which were inoculated by extraneural routes. This property, which has been observed by others(5,10), suggested the utility of these strains for the rapid assay of anti-Lansing type poliomyelitis antibodies. In a preliminary experiment it was found that the injection of 0.25 ml of gamma globulin in one flank of mice followed immediately by injection into the other flank of 0.25 ml of a 10% suspension of the nervous tissues harvested from the 121st hamster passage of the B series left 6 of 6 mice unaffected, while 6 control mice died when given virus alone. Table III summarizes the results of an experiment designed to test the duration of the passive immunity induced in weanling mice by administration of immune serum globulin of placental origin, and Table IV gives the results of a titration of a sample of commercial immune serum globulin for antibody content. The results illustrate the utility of this strain of virus for assay purposes and at the same time indicate the antigenic affinity of the passage virus for antibodies which occur in commercially available immune serum globulin.

Discussion. Continuous passage of the MEF1 strain (Lansing type, Type 2) of poliomyelitis virus in suckling hamsters by the intracerebral route has resulted in the development of two substrains of virus that exhibit properties which are markedly different from those of the parent strain. In conformity with the observations of others(3,5) who have worked with the MEF1 strain of poliomyelitis virus in young animals, it was noted that the incubation period shortened and that the concentration of virus in the nervous tissues increased with progressive serial passages in suckling hamsters. By harvesting the brain and cord tissues as early as 24 hours after inoculation, when the first symptoms of illness appeared, titers as high as $10^{-6.5}$ to $10^{-7.0}$, or even greater, were attained with considerable regularity. Virus concentrations of this magnitude have greatly facilitated the development of a complement-fixing antigen and an inactivated or killed virus vaccine, both of which are under investigation at the present

TABLE III. Protective Action in Mice of Immune Serum Globulin (Placental Origin) Against the MEF1-B Strain Poliomyelitis Virus.

Interval between treatment and challenge	Mortality ratio* — Treated mice	Mortality ratio* — Untreated mice
24 hr	1/10	6/8
48	0/10	9/10
72	1/10	4/10
96	0/10	8/10
7 days	0/10	7/10
14	0/9	8/10
28	4/6	5/8

* Mortality ratio = No. of mice developing symptoms/No. surviving without symptoms.

TABLE IV. Neutralization Test in Mice with MEF1-A Virus and Immune Serum Globulin.

Material inj.	Globulin dilution	LD_{50} titer of virus
Virus* + immune serum globulin†	1:4	$10^{-1.83}$
	1:8	$10^{-2.68}$
	1:16	$10^{-3.17}$
	1:32	$10^{-2.68}$
	1:64	$10^{-3.81}$
	1:128	10^{-3}
	1:256	$10^{-3.15}$
	1:512	$10^{-3.03}$
Virus* + saline		$10^{-6.17}$
Virus* + normal rabbit serum‡		$10^{-5.32}$

* Virus = 110th hamster passage A series.

† Immune serum globulin = Lederle Lot #2167-82A.

‡ Normal rabbit serum contained 1:10000 merthiolate.

time. In this connection it should be noted that our data agree in general with those reported recently by Casals *et al.*(11,12).

The B series substrain of the MEF1 virus has a remarkably high degree of virulence for 2- to 4-day-old suckling hamsters as well as Swiss white mice, but it apparently has lost in great measure its pathogenicity for the Rhesus monkey. While the feeding experiment in chimpanzees with the B substrain of virus was exploratory only, it is of interest to note that in the absence of clinical signs of disease or demonstrable virus in their feces, both chimpanzees developed neutralizing antibodies for the A substrain hamster passaged MEF1 virus. Furthermore, when subsequently fed the heterotypic Brunhilde virus (Type 1) and the homotypic Wallingford virus (Type 2), both chimpanzees became symptomless excretors of these virus strains and showed an anamnestic antibody response for MEF1-A substrain of virus. Should the extensive work now in progress confirm the preliminary evidence that the B substrain of hamster passaged MEF1 virus is avirulent for primates, that it does not induce a carrier state but does immunize when administered by the oral route, material progress will have been made towards producing a practical living virus vaccine.

The high LD_{50} titers attained in suckling hamsters by these two substrains of MEF1 virus may be assumed to favor the appearance and capture of variants differing from those of the original virus clone(13) when methods appropriate for the demonstration of such variants are used. In the accompanying papers by Roca-Garcia(14) and Cabasso(15) *et al.* evidence is presented which suggests that a mutant of poliomyelitis virus capable of growth in the chick embryo appeared between the 109th and the 115th hamster passage of the MEF1-A virus. This evidence of broadened host range gives additional promise of the eventual development of an immunizing agent which may be both immunogenically effective and clinically acceptable.

Summary. 1. The MEF1 strain of poliomyelitis virus (Lansing type, Type 2) has been adapted to suckling hamsters using the intracerebral route of inoculation, whereby two substrains of virus have been developed which show a much shortened incubation period and a markedly increased concentration of virus in the nervous tissues. The strains adapted to suckling hamsters have been found to cause paralysis after intramuscular or intraperitoneal inoculation into 21- to 28-day-old mice. 2. Intracerebral inoculations in 20 Rhesus monkeys suggest the MEF1-B substrain of virus has become somewhat reduced in virulence for primates. Two chimpanzees which were fed nervous tissue suspensions of the B substrain remained symptom-free, developed neutralizing antibodies and failed to excrete virus in their stools.

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Poliomyelitis. II. Propagation of MEF1 Strain of Poliomyelitis Virus in Developing Chick Embryo by Yolk Sac Inoculation.* (19928)

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Since Armstrong's discovery in 1939(1) that a strain of poliomyelitis virus, now known as Lansing or Type 2 virus, may be transmitted to cotton rats, several investigators have been successful in adapting other Lansing type strains(2-5) and a Leon, Type 3 virus(6) to rodents. Recently, our laboratory(7) has succeeded in serially infecting suckling hamsters with the MEF1 strain Lansing type poliomyelitis virus by the intracerebral route, thereby bringing about a considerable increase of its LD_{50} titer for mice. While our work was in progress, Casals *et al.*(8,9) and Selzer and coworkers(10) reported the adaptation of the MEF1 strain Lansing type virus to newborn mice. Although the increase of its LD_{50} titer was not of the same magnitude as that obtained in suckling hamsters(7), the newborn mouse-adapted virus has yielded a diagnostic antigen for the complement-fixation test. The more ready transmissibility of the Lansing type strains of virus to rodents has been used to differentiate this group of viral agents from those of the Brunhilde or Leon types(11). Furthermore, the inability of any of the known types or strains of human poliomyelitis virus to grow in the developing chick embryo has been considered a characteristic distinguishing these viruses from other neurotropic agents(12). Of the several fruitless attempts made to adapt human poliomyelitis viruses to the developing chick embryo, those of Burnet(13), Stimpert(14) and Kast and Kolmer(15) may be cited. Gard(16) was able to infect a monkey by injecting it with the brain tissue of chick embryos inoculated ten days previously with a strain of poliomyelitis virus, but obtained no evidence of true virus multiplication. Schultz and Enright(17,18) reported the cultivation in the chick embryo of the SK, MM and C(M) strains of so-called "murine poliomyelitis" virus, now recognized as strains of encephalomyocarditis virus(19). Schultz and Enright also adapted Theiler's GDVII strain of mouse encephalomyelitis virus to the chick embryo, thus confirming the work of Gard, Dunham and Parker(20,21). They failed, however, to propagate 5 strains of human poliomyelitis virus in the chick embryo. More recently, Enders(22) and his associates reported failure to propagate a Lansing type strain of poliomyelitis virus in chick, beef or mouse embryonic tissue or in the testicular tissue of the rabbit, whereas the agent was readily propagated in cultures of human or monkey tissues.

The purpose of this communication is to present experimental evidence of the propagation of the MEF1 strain of poliomyelitis virus by the yolk sac route of inoculation in the developing chick embryo, following its adaptation to suckling hamsters.

Materials and methods. Virus strains: The virus used to initiate the 2 embryo passage series described below represented the 119th hamster passage of the MEF1-A strain of Moyer *et al.*(10). In the mouse neutralization tests either the 64th or 131st hamster passage of Moyer's MEF1-A strain was employed for the determination of antibodies in the experimental monkey sera. The challenge virus used to test the immunity of monkeys was a pooled monkey cord suspension prepared from the 4th monkey passage of the Armstrong Lansing strain, which was obtained in its 197th mouse passage through the courtesy of Dr. Howard A. Howe, Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md. This virus is referred to in the text as pooled monkey cord Lansing virus.

Chick embryos and passage method: Unless otherwise stated, 7-day-old chick embryos were incubated for 7 or 8 days at 37°C after yolk sac inoculation with an 0.6 ml dose of

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a 66% tissue suspension. Embryos were harvested and homogenized in a Waring blendor with 3 ml of buffered saline per embryo, giving an approximate 66% tissue suspension, referred to hereafter as undiluted material. The resulting tissue suspension was centrifuged for 10 minutes at 1500 rpm in a No. 1 International angle head centrifuge. The supernate was used for chick embryo passage and for the inoculation of test animals only when found to be bacteriologically sterile.

Immune sera: A Lansing type immune serum prepared in our laboratory by hyperimmunization of a monkey which survived intracerebral inoculation with the WW strain of virus(23) was commonly used. This Lederle serum has been employed routinely as a standard Lansing type immune serum and its specificity has been repeatedly demonstrated. In addition, for critical neutralization tests, a Y-SK immune monkey serum was obtained through the courtesy of Dr. Joseph L. Melnick of Yale University, New Haven, Conn., and a Lansing (Armstrong) immune serum was received from Dr. Howe. A GDVII immune serum prepared in rabbits immunized by 25 weekly injections of GDVII infected mouse brain suspensions was also employed.

Neutralization tests: The neutralization test procedure was carried out as follows: Undiluted normal and immune sera, respectively, were mixed with serial dilutions of the virus under test. The mixtures were incubated at 37°C for 90 minutes before intracerebral injection in mice. Neutralization indices were calculated on the basis of the difference between the log LD_{50} titers obtained with normal and immune serum, and are expressed as the number of LD_{50} doses of virus neutralized by undiluted serum.

Test animals: Swiss albino mice 28 to 35 days old were used for virus titrations and serum neutralization tests. Rhesus monkeys (*Macaca mulatta*) weighing 7 to 8 pounds were employed for virulence tests and virus titrations.

Experimental. Chick embryo series R-302. This series was initiated in chick embryos on February 4, 1952, with a 10% cord suspension prepared from the second mouse brain passage of the 119th hamster passage of Moyer's MEF1-A Lansing type poliomyelitis virus. Live embryos were harvested 4 days later and a 66% suspension prepared and injected intracerebrally at once into 3 Swiss albino mice. Two days later a second group of 2 mice were similarly inoculated with the remainder of the chick embryo suspension, which had been stored in the meantime at 4°C. All 5 mice of the 2 groups became paralyzed 4 to 6 days after inoculation. A 10% suspension was prepared from the pooled cord tissues of the paralyzed mice of these 2 groups, and chick embryos were inoculated by the yolk sac route with the cord suspension. The embryos were harvested on the 7th day, homogenized and inoculated into 4 additional mice. Separate brain and spinal cord suspensions were prepared from the 4 mice, all of which became paralyzed. Separate serial egg passages labeled R-302-cord (R-302-C) and R-302-Brain (R-302-B) were made from the indicated mouse tissues on March 3, 1952. Live embryos were harvested from each egg passage on the 4th and again on the 7th day after inoculation. Pending infectivity tests in mice, the remaining portions of the harvested chick embryo tissue suspensions were shell frozen and held at —30°C for 7 days. The mouse tests demonstrated the presence of virus in embryos harvested at 7 days both in egg passages R-302-C and R-302-B. The frozen reserve suspensions from these two, first chick embryo passages were therefore pooled to initiate the second chick embryo passage R-302-2 in 26, 7-day-old chick embryos. Thereafter, this strain (R-302) has been maintained in serial uninterrupted chick embryo passages for 41 consecutive generations. Groups of 4 to 7 mice were injected intracerebrally with undiluted chick embryo suspensions at each passage level. The inoculated mice invariably developed paralysis and died. Tissue suspensions from the 8th, 9th, 11th and 13th and all subsequent chick embryo passages were titrated intracerebrally in mice. The results of these titrations are presented in the accompanying chart.

Neutralization tests for identification of the passage virus were performed in mice with chick embryo tissue suspensions, or with brain and cord tissue suspensions of mice which had

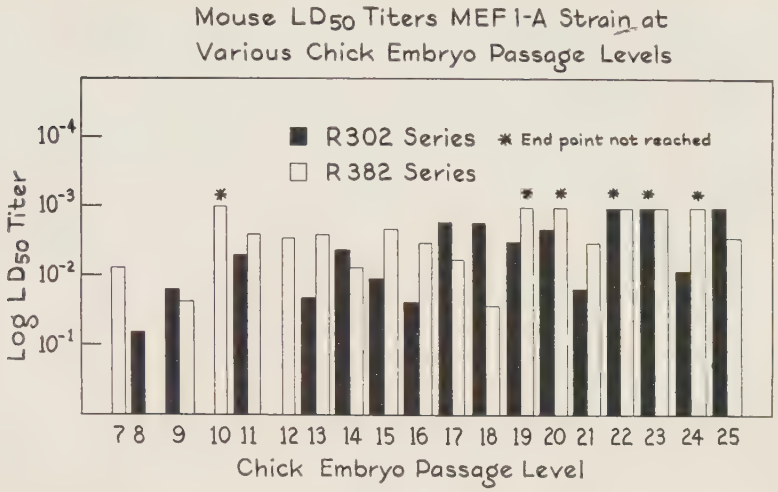

FIG. 1.

been injected intracerebrally either with chick embryo suspensions, or with cord tissue taken from monkeys that had been injected intracerebrally with chick embryo suspensions. The results of the tests done at the 7th, 9th, 10th, 12th and 21st chick embryo passage levels of the R-302 series are summarized in Table I. The data show that the viral agent under test was appreciably neutralized by the Lansing type immune monkey serum prepared in our laboratory, as well as by those sera received from Dr. Joseph Melnick and Dr. Howard Howe of Yale and Johns Hopkins Universities, respectively. These findings strongly indicate the identity of the R-302 series virus as Lansing type human poliomyelitis virus.

The identification of the virus was further supported by the results obtained in virulence tests in Rhesus monkeys. On May 9, 1952, 2 Rhesus monkeys (2135 and 2136) were inoculated with a 66% chick embryo suspension of the 7th consecutive egg passage of the R-302 series. Monkey 2135 was injected intracerebrally with 0.5 ml of this suspension, while monkey 2136 was inoculated intramuscularly with 1.0 ml of the same material. Seven days later, the monkey injected intracerebrally (2135) showed signs of weakness of the right leg, accompanied by noticeable general tremor. The next day the animal showed complete paralysis of both legs and right arm. Later that day, it was sacrificed and its spinal cord removed. Part of the spinal cord was fixed in 80% alcohol for histopathologic examination† and the remainder was made into a 10% tissue suspension, which was inoculated intracerebrally into 8 Swiss mice; 7 of these became paralyzed between the 8th and 15th day after inoculation.

A suspension of the brain and spinal cord tissues from 2 of these paralyzed mice was tested by neutralization procedure against normal monkey serum and the Lederle anti-Lansing monkey serum. The results, shown in Table I, strongly suggested that the passage virus was a Lansing type strain of human poliomyelitis virus.

Monkey 2136 remained symptom-free following intramuscular injection and serum obtained 68 days after inoculation failed to show neutralizing antibodies.

A tissue suspension having a mouse LD_{50} titer of $10^{-2.3}$, prepared from the 11th chick embryo passage of the R-302 series was titrated intracerebrally in monkeys. The undiluted tissue suspension and 10^{-1} and 10^{-2} dilutions thereof were injected intracerebrally in duplicate monkeys (Table II). Only one of the 6 monkeys (2245) developed symptoms of poliomyelitis and it recovered.

† Dr. George A. Jervis, who performed the histopathologic study, reported typical cord lesions of poliomyelitis.

TABLE I. Chick Embryo Adapted MEF1 Strain of Poliomyelitis Virus. Results of neutralization tests.

Passage series	Virus passage level	Mouse LD_{50} titers and neutralization indices	Serum used: Normal monkey	Immune (Lederle)	Immune (Y-SK)*	Immune (Armstrong)†	Immune (GDVII)
	7 E + 1 monkey (2135) + 1 mouse	LD_{50}	$10^{-3.5}$	$10^{-2.2}$			
		N.I.		47			
R-302	9 E + 1 mouse	LD_{50}	$10^{-2.66}$	$10^{-1.5}$			
		N.I.		20			
	10 E + 1 mouse	LD_{50}	$10^{-3.2}$	$<10^{-1.3}$			
		N.I.		>97			
	12 E + 1 mouse	LD_{50}	$10^{-3.3}$	$10^{-1.7}$	$10^{-1.3}$		
		N.I.		74	100		
	21 E	LD_{50}	$10^{-1.7}$			10^{-0}	
		N.I.				>84	
	7 E + 1 mouse	LD_{50}	$>10^{-3.0}$	$10^{-1.0}$			
		N.I.		>100			
	9 E + 1 mouse	LD_{50}	$>10^{-3.8}$	$10^{-2.0}$			$>10^{-3.8}$
		N.I.		63			0
R-382	11 E + 1 monkey (2312) + 1 mouse	LD_{50}	$10^{-3.4}$	$10^{-1.6}$			
		N.I.		63			
	17 E	LD_{50}	$10^{-2.0}$			10^{-0}	
		N.I.				>100	

7 E + 1 monkey + 1 mouse = 7 embryo passages + 1 monkey brain passage + 1 mouse brain passage used as mouse brain suspension.

9 E + 1 mouse = 9 embryo passages + 1 mouse brain passage used as mouse brain suspension.

21 E = 21st embryo passage used as embryo suspension.

* Lansing immune monkey serum received from Dr. Joseph L. Melnick of Yale University.

† " " " " " " Dr. Howard A. Howe of Johns Hopkins University.

The sera of monkeys 2245 and 2246, both of which had received the undiluted inoculum, as well as the serum of monkey 2250, which had received the 10^{-2} dilution intracerebrally, were shown to contain neutralizing antibodies by a qualitative test carried out with a virus suspension derived from the 64th hamster passage of Moyer's MEF1-A strain. In a subsequent quantitative neutralization test carried out with Moyer's 131st hamster passage of the MEF1-A strain of virus, neutralization indices of 31,620 and 39,810 were obtained for the sera of monkeys 2245 and 2246, respectively. The serum of monkey 2250 was not tested quantitatively. All 3 monkeys (2245, 2246 and 2250) were challenged intracerebrally with Lansing monkey cord pool virus 68 days after original inoculation, and all 3 survived without showing any symptoms of poliomyelitis. In contrast, all of the 13 normal, control monkeys used in the test came down with typical poliomyelitis 7 to 15 days after intracerebral injection.

The 11th chick embryo passage virus of the R-302 series was shown thereby to induce in monkeys the formation of antibodies which neutralized the MEF1-A strain virus in significant degree and produced in such animals a resistance capable of withstanding direct intracerebral challenge with at least 700 monkey MLD's of virulent virus of the Lansing monkey cord pool.

The remaining 3 monkeys used in the titration (2247, 2248 and 2249) apparently failed to develop either symptoms or antibodies and were not challenged intracerebrally.

Chick embryo series R-382. This series was initiated in 7-day-old chick embryos inoculated by way of the yolk sac on February 13, 1952, with a 10% brain and cord suspension prepared from the 3rd mouse passage of Moyer's 119th hamster passage of the MEF1-

TABLE II. Identification of the Chick Embryo Propagated Virus by the Response of Monkeys to Inoculation.

Series No.	Chick embryo passage	Monkey No.	Dilution used	LD_{50} titer in mice	Vol., ml	Route	Date	Symptoms	Monkey challenge		Mouse neutralization tests	
									I.C.	Date	Qualitative M/R‡	Quantitative N.I.§
R-302	7	2135	Undil.	N.D.	.5	I.C.	5/9/52	Yes*	—		—	—
		2136	"	"	1	I.M.	"	No	N.D.		7/8	—
R-382	9	2245	"	$10^{2.30}$	.5	I.C.	6/5/52	Yes†	1'	8/12/52	0/8	31620
		2246	"	"	"	"	"	No	1'		0/8	39810
	11	2247	10^{-1}	"	"	"	"	"	N.D.		8/8	—
		2248	"	"	"	"	"	"	N.D.		8/8	—
		2249	10^{-2}	"	"	"	"	"	N.D.		8/8	—
		2250	"	"	"	"	"	"	1'		0/7	—
	9	2253	10^{-1}	$10^{-1.64}$	"	"	"	"	1'	8/12/52	0/8	21380
		2254	"	"	"	"	"	"	1'		7/8	631
	11	2312	Undil.	N.D.	"	"	6/30/52	Yes*	—	8/12/52	—	—
		2313	"	$10^{2.87}$	"	"	"	No	1'		3/8	25120

13 normal, control monkeys inj. intracer. with Lansing monkey cord pool virus on 8/12/52; all developed typical poliomyelitis within 7 to 15 days.‖

I.C. = Intracerebral; I.M. = Intramuscular; N.D = Not done; P = Protected.
* Weakness followed by paralysis of the extremities 7 days after inoculation. Lansing type virus recovered from the cord. Histologically typical poliomyelitis.
† General tremors and right arm weakness 7 days after inoculation. Monkey recovered after 2 weeks.
‡ Qualitative neutralization tests performed with the 64th hamster passage of the MEF1-A strain of virus, expressed as the mortality ratio = M/R.
§ Quantitative neutralization tests performed with the 131st hamster passage of the MEF1-A strain of virus, expressed as the neutralization index = N.I.
‖ Challenge dose represented 700 MLD's of the Lansing pooled monkey cord tissue.

A strain. The live embryos were harvested 7 days later, and a 66% tissue suspension prepared from them was inoculated intracerebrally in a group of 4 mice designated R-382. From the paralyzed mice of R-382 a 10% suspension of pooled brain and spinal cord tissue was prepared and injected into the yolk sacs of 13, 7-day-old chick embryos on March 17, 1952. Since that date the R-382 series has been maintained continuously in chick embryos by yolk sac inoculation for 38 generations and the passage series is still in progress. Groups of 3 to 7 mice were injected intracerebrally with tissue suspensions at each passage level as a qualitative test for the presence of virus. The inoculated mice invariably developed paralysis and died. Tissue suspensions from the 7th, 9th and all subsequent chick embryo passages were titrated intracerebrally in mice. The results of these titrations are presented graphically in the accompanying figure.

Neutralization tests for identification of the passage virus were done with tissue suspensions representing the 7th, 9th, 11th and 17th chick embryo passages. The results, summarized in Table I, show that the virus at the first 3 passage levels of this sequence was neutralized by Lederle anti-Lansing monkey serum and that the virus at the 17th chick embryo passage was neutralized by the anti-Lansing monkey serum received from Dr. Howe. In addition, the 11th embryo passage virus was not neutralized by the immune serum prepared against the GDVII strain of mouse encephalomyelitis virus. These neutralization tests indicate that the R-382 series chick embryo passage virus is a Lansing type strain of human poliomyelitis virus.

This identification is substantiated by the observations made on monkeys 2312 and 2313 (Table II), which were injected intracerebrally with a 66% chick embryo tissue suspension having a mouse LD_{50} titer of $10^{-2.6}$ prepared from the 11th chick embryo passage. Monkey 2312 showed typical paralytic symptoms on the 7th day after inoculation. It was sacrificed the following day and sections of its spinal cord revealed typical poliomyelitic lesions.† Virus recovered from the cord of this animal was neutralized by Lederle anti-

Lansing monkey immune serum (Table I). Monkey 2313 survived without the development of symptoms, but a postinoculation serum sample showed a neutralization index of 25,120 when tested against Moyer's 131st hamster passage of MEF1-A strain virus.

Similarly, monkeys 2253 and 2254, which were injected intracerebrally with 0.5 ml each of a 6% chick embryo suspension of the R-382 series 9th passage, failed to show any signs of illness but developed neutralization indices of 21,380 and 631, respectively, when tested against Moyer's 131st hamster passage MEF1-A virus. These 3 monkeys (2253, 2254 and 2313) were later challenged intracerebrally (Table II) with Lansing monkey cord pool virus and all 3 survived without showing any symptoms of poliomyelitis, whereas all of 13 normal, control monkeys used in the test developed typical poliomyelitis within 7 to 15 days.

The chick embryo passage virus of the R-382 series was thus shown to parallel that of the R-302 series at the same passage levels. It produced typical symptoms and cord lesions of poliomyelitis in inoculated monkeys; it stimulated the formation of antibodies against the MEF1-A strain virus and induced resistance in monkeys to intracerebral challenge with virulent Lansing type monkey cord pool virus.

Observations on the passage virus in chick embryos. After approximately 40 serial passages in the chick embryo the virus neither kills infected embryos nor causes superficial gross lesions by which its presence and multiplication can be judged.

Using the yolk sac route of inoculation and titrating tissues and fluids 6 to 8 days later, the highest concentrations of virus have been found in the body of the embryo. Testing of tissue suspensions prepared from decapitated embryos gave virus titers comparable to those obtained with complete embryos, which suggests that virus multiplication is not restricted to the nervous tissues. Only traces of virus have been demonstrated in yolk sac or allantoic membrane suspensions.

Attempts to adapt the MEF1-A strain of virus to the chick embryo at the 70th, 80th, 100th and 109th hamster passage levels have not been successful to date. The 115th hamster passage virus, however, has now been established in chick embryos. Thus the experience in adapting the MEF1 strain of poliomyelitis virus to the chick embryo closely parallels that of Schlesinger(24) in adapting dengue virus to growth in the chick embryo.

These observations suggest that between the 109th and the 115th hamster passage a virus mutant appeared which was able to grow in the chick embryo. Such an interpretation is also consistent with the apparent failure of other investigators to adapt other strains of human poliomyelitis virus to growth in this medium.

Summary. 1. The MEF1-A strain of human poliomyelitis virus(10), after 119 serial passages in suckling hamsters, has been maintained in one passage series for 41 consecutive generations and in a second series for 38 generations in the developing chick embryo by the yolk sac route of inoculation. Both lines of passages are still in progress. 2. The identity of the chick embryo adapted virus with human poliomyelitis virus was established by mouse neutralization tests in which anti-Lansing type immune sera from 3 different sources were used; by the ability of the passage virus to stimulate the formation of antibodies in monkeys which neutralize known virulent strains of Lansing type poliomyelitis virus; and by the ability of monkeys to withstand intracerebral challenge with known virulent strains of poliomyelitis virus after they had survived inoculation with the chick embryo passage virus. The identity of the passage virus was further established by its ability to induce typical symptoms and histopathologic lesions in monkeys following intracerebral inoculation.

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Poliomyelitis. III. Propagation of MEF1 Strain of Poliomyelitis Virus in Developing Chick Embryo by Allantoic Cavity Inoculation.* (19929)

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The numerous unsuccessful attempts to propagate poliomyelitis virus in the developing chick embryo have led investigators to consider this viral agent as being uncultivable in this experimental host and to use this characteristic as a distinguishing feature between poliomyelitis and other neurotropic agents(1). Similar unsuccessful results had repeatedly been obtained with viruses other than poliomyelitis which later, however, were successfully grown in the chick embryo. Such, for example, was the case with canine distemper virus which was propagated in fertile hen's eggs almost simultaneously by Haig(2) in South Africa and by two of us(3) in this country. Equally successful efforts are now being reported with a Lansing type strain of poliomyelitis virus. In the two preceding papers of this series, Moyer and coworkers (4) have described the serial passage of the MEF1 strain of poliomyelitis virus in the suckling hamster with a resulting increase of its LD_{50} titer for mice, and Roca-Garcia and coworkers(5) have reviewed the pertinent literature and reported the propagation of Moyer's MEF1-A strain in the developing chick embryo by the yolk sac route of inoculation. The present communication describes the propagation of Moyer's hamster-adapted virus by inoculation into the allantoic sac of the developing chick embryo. The experimental work reported here was conducted independently, with different personnel and in separate quarters.

Materials and methods. Virus strains. The

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strain employed in this study was the MEF1 Lansing type of poliomyelitis virus, which has been serially passed in unweaned hamsters by one of us(4) and designated as MEF1-A. The preparations used for egg inoculation were 20% suspensions of baby hamster brain representing the 131st serial hamster passage which had a mouse LD_{50} titer of $10^{-6.52}$. The MEF1 strain(6) which was adapted to suckling mice (7), and for which we are indebted to Dr. Jordi Casals of the Rockefeller Foundation Laboratories, New York, N. Y., was used in the cross-neutralization tests on monkey sera.

Chick embryos and passage method. The chick embryos employed were 7 days old when inoculated in the allantoic cavity with 0.25 ml of either infected hamster brain or chick embryo tissue suspensions. Inoculated eggs were incubated at 37°C first for 7 days and in later egg passages for 4 to 5 days. Twenty per cent tissue suspensions were routinely employed for passage. *Mice.* Three-week-old Swiss albino mice weighing 10 to 12 g each were used for titration and standard neutralization tests.

Immune sera. The immune and normal sera used for the identification of the chick embryo propagated virus were derived from either hamsters or monkeys. The following sera were inactivated in the water bath at 56°C for 30 minutes before their use in this study: 1) an immune hamster serum derived from animals hyperimmunized with the hamster propagated MEF1-A virus at its 86th passage in hamsters; 2) immune monkey sera against either Brunhilde, Leon or Lansing A types of virus, received through the courtesy of Dr. Howard A. Howe, Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md., to whom we are very grateful; 3) immune monkey sera against either Brunhilde, Leon or Y-SK Lansing type virus, very kindly sent to us by Dr. Joseph F. Melnick, Yale University, New Haven, Conn.; and 4) a normal hamster serum which was used as control.

Neutralization tests. Identification of the virus adapted to chick embryo passage was based on standard neutralization tests. Equal volumes of undiluted serum and serial tenfold dilutions of infected chick embryo suspensions were mixed, incubated in a water bath for one hour at 37°C and inoculated into mice by the intracerebral route. The mice were observed for 21 days and the LD_{50} titers were calculated according to the Reed and Muench formula(8). Neutralization indices were derived from the differences between the log LD_{50} titers of normal and immune serum mixtures.

Experimental. Adaptation of the virus to the developing chick embryo. Of the numerous attempts made in this laboratory to propagate poliomyelitis virus in the chick embryo by using different routes of inoculation, different incubation temperatures and embryos of different ages, the following series was successfully carried through at least 40 serial chick embryo passages: On April 15, 1952, a 20% suspension of suckling hamster brain representing the 131st serial passage of the MEF1-A virus(4) was injected in the allantoic cavity of 8, 7-day-old embryos, each embryo receiving 0.25 ml volume. These eggs, which constituted the first chick embryo passage of the present series, were incubated at 37°C for 7 days. At the end of this incubation period embryos and chorio-allantoic membranes of inoculated eggs were harvested aseptically, pooled, ground in a Waring blendor and centrifuged at 2,000 rpm for 20 minutes in a size 1 International anglehead centrifuge. The undiluted suspension was immediately titrated in tenfold dilutions in mice. Four mice were used for each dilution and each mouse received 0.03 ml intracerebrally of the respective dilution. A 20% suspension was in turn made from the undiluted material of the first chick embryo passage and inoculated via the allantoic cavity into 8, 7-day-old chick embryos and these also were incubated at 37°C for 7 days. Harvesting and mouse titration of material from this second passage and from the 8 succeeding passages were made in a manner identical to that used for the first passage.

Meanwhile, having found by mouse titrations that a 4- to 5-day incubation period at 37°C yielded higher concentrations of virus in the embryonic tissues than did a 7-day incubation period, the shorter time interval was used with chick embryo passages higher

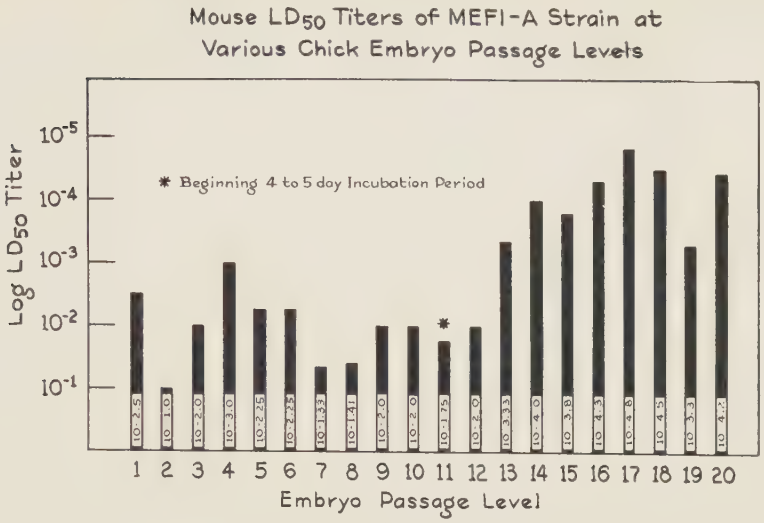

FIG. 1.

than the 10th. Methods of harvest, mouse titration and egg inoculation, however, remained the same as those used in earlier passages. At the time of writing, 40 successive passages of this virus in chick embryos have been made. It may be noted in the accompanying chart that beginning with the 13th passage in chick embryos the mouse LD_{50} titers increased, reaching a maximum of $10^{-4.8}$ at the 17th chick embryo passage level.

Identity of the passage virus. Virus tissue suspensions representing the 6th, 13th and 16th chick embryo passages were employed in standard neutralization tests. The sera used in these tests were: 1) an immune hamster serum against the parent MEF1-A strain of virus; 2) immune monkey sera against the homotypic Armstrong and Y-SK strains of Lansing virus; and 3) immune monkey sera against the heterotypic Brunhilde and Leon virus strains. The results of the neutralization tests are summarized in the accompanying table. The data indicate that the passage virus propagated serially by the allantoic cavity route in the developing chick embryo is a *bona fide* strain of Lansing type poliomyelitis virus. The passage virus was not only neutralized by an immune hamster serum prepared against the MEF1-A strain of Moyer, but it was also neutralized by immune monkey sera prepared in other laboratories against homotypic Lansing type strains. A slight degree of neutralization may be observed also in the tests of the 13th and 16th passage level virus in the presence of one of the Brunhilde and both of the Leon immune monkey sera. It is difficult to assess the significance of this slight heterotypic neutralization, but in light of the recent observations of Casals *et al.*(9,10) and Sabin(11), it is suggestive of antigenic overlapping within the group of poliomyelitis viruses.

Virulence tests in monkeys and chimpanzees. A 50% chick embryo tissue suspension having a mouse LD_{50} titer of $10^{-3.3}$ was prepared from the 13th passage and used in a preliminary test of virulence in Rhesus monkeys. Two monkeys (2441 and 2442) each received 0.5 ml intracerebrally, and two others (2443 and 2444) were injected intramuscularly with 3.0 ml. One of the intracerebrally injected monkeys (2442) developed weakness of the hind legs and tremor beginning on the 9th day after inoculation, but recovered completely 9 days later. Monkey 2444 died of tuberculosis 19 days after intramuscular injection. The other two monkeys remained free of nervous symptoms. Serum samples obtained from the 3 surviving monkeys 35 days after injection were tested for antibodies by mouse neutralization tests. Against Casals' suckling mouse propagated MEF1 strain of virus(7),

TABLE I. Results of Serum-Neutralization Tests in Mice with Chick Embryo Adapted MEF1-A Virus and Immune Sera Against Lansing, Brunhilde and Leon Types Viruses.

Egg passage level	Results	Serum-virus mixtures: Lansing immune sera: MEF1 hamster	Lansing immune sera: Lansing A monkey*	Lansing immune sera: Y-SK monkey†	Brunhilde immune sera: Monkey*	Brunhilde immune sera: Monkey†	Leon immune sera: Monkey*	Leon immune sera: Monkey†	Normal control: Hamster
6	LD_{50} titer	$<10^{-1}$	—	—	—	—	—	—	$>10^{-4.5}$
	N‡	>3162							
13	LD_{50} titer	$<10^{-1}$	$<10^{-1}$	—	$10^{-2.5}$	—	$10^{-3.3}$	—	$10^{-4.7}$
	N‡	>5012	>5012		158		25		
16	LD_{50} titer	$10^{-2.1}$	—	$10^{-1.6}$	—	10^{-6}	—	10^{-4}	$10^{-5.5}$
	N‡	2512		7943		0		32	

* Supplied by Dr. Howard A. Howe, Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md.

† Supplied by Dr. Joseph F. Melnick, Yale University, New Haven, Conn.

‡ N = Neutralization index.

the serum of one of the intracerebrally injected monkeys (2442) had a neutralization index of 316, while that of its mate (2441) was only 21. The serum of the surviving intramuscularly injected monkey (2443) had an index of only 25. The complement-fixing titers of these 3 sera were found to parallel their neutralization indices. Thus, monkey 2442 showed a complement-fixing titer of 4+ at 1:16 serum dilution; monkey 2441 showed 3+ at 1:2 dilution; and monkey 2443 failed to give a significant complement-fixing response.

A 20% chick embryo tissue suspension of the 17th passage having a mouse LD_{50} titer of $10^{-4.8}$ was titrated intracerebrally in duplicate monkeys. A dose of 0.5 ml of the undiluted suspension, or of 10^{-1}, 10^{-2} or 10^{-3} dilutions thereof, was injected. During the 21-day observation period none of the 8 monkeys showed paralytic or other nervous symptoms. Serum samples from these animals are to be assayed for neutralizing antibodies and the monkeys are to be tested for active immunity by intracerebral challenge. The same virus-tissue suspension was fed to 2 chimpanzees and injected intramuscularly into 2 additional chimpanzees in an attempt to determine its pathogenicity and antigenicity by the oral or intramuscular routes. All 4 chimpanzees have remained normal to date, at least 3 months after exposure. Results of the neutralization tests performed in mice with the 14th, 28th, 42nd and 56th day serum samples of the 4 chimpanzees against both the parent MEF1 and the Y-SK strains of virus, indicate the presence of neutralizing antibodies in the serum of all 4 animals against both virus strains. Details of the final results will be reported in a later communication.

Adaptation of the MEF1-B Moyer strain to the chick embryo. Using the technic described above, the MEF1-B Moyer strain of suckling hamster virus was also adapted to grow in the developing chick embryo and, at the time of writing, is in its 23rd egg passage. Mouse LD_{50} titers increased from $10^{-4.0}$ at the first egg passage level to greater than $10^{-7.0}$ at the 7th passage. A neutralization test in mice with the 6th chick embryo passage virus and the homologous immune hamster serum clearly shows the identity of this virus with the Lansing type poliomyelitis strain.

Discussion. Because of the many unsuccessful attempts made to cultivate poliomyelitis virus in the developing chick embryo, most investigators were led to believe that this group of agents was not able to parasitize this host and that this characteristic could be used to distinguish poliomyelitis virus from other neurotropic agents(1). One of us(12), however, expressed his confidence that someone would eventually succeed in adapting poliomyelitis virus to the chick embryo. As reported above, the present authors have been successful in serially propagating both of Moyer's(4) MEF1-A and MEF1-B hamster passaged virus strains in the chick embryo, with significant increases of their mouse LD_{50} titers in the higher serial embryo passages.

Whether this adaptation was made possible by the increased titers obtained in the serial hamster passages is difficult to determine at this time, but this point is being investigated. The adaptation to the chick embryo of this Lansing type poliomyelitis virus offers new promise both in the field of diagnosis as well as of prophylaxis against the disease. Tests already carried out in monkeys strongly indicate that the chick embryo-propagated virus has lost in great measure its virulence for primates, inducing no severe disease symptoms even when administered intracerebrally in comparatively massive doses. Furthermore, accumulating evidence obtained in this laboratory as well as by other workers(9-11) shows a certain antigenic overlapping between the Lansing type and the Brunhilde and Leon types of poliomyelitis agents. This gives reason to hope that immunity engendered by one type of virus may also elicit an adequate basic immunity against heterotypic strains whereby some of the difficulties encountered in diseases with a plurality of strains may be overcome. It is also evident that the use of the developing chick embryo as a source of virus infected tissues may obviate many of the dangers and difficulties encountered in the use of infected rodent brain suspensions or tissue culture preparations requiring mammalian tissues.

The oral administration of a living, modified virus, such as one described here, seems to offer the most logical approach as a prophylactic measure against poliomyelitis(13). Whether a single oral dose would be sufficient for the adequate production of both homo- and heterotypic antibodies will have to be determined by further experimental work which is being carried out.

Summary and conclusions. Data describing the cultivation of the MEF1 Lansing type poliomyelitis virus in the developing chick embryo are reported. This strain of poliomyelitis virus was derived from the original MEF1 strain following its adaptation to suckling hamsters. Neutralization tests performed at three passage levels with *bona fide* immune hamster and monkey sera prepared against three homotypic strains of the Lansing type virus point to the identity of the chick embryo adapted and propagated virus with this type of poliomyelitis agents. A moderate neutralization has also been shown to take place between the chick embryo adapted virus and immune monkey Brunhilde and Leon sera, but, as indicated, it is difficult to attribute much significance to this finding at the present time. Some experimental evidence was obtained indicating that the chick embryo adapted virus does not induce paralysis or death in Rhesus monkeys even following intracerebral inoculation with comparatively massive doses of virus. The effect of this virus on the immune response of Rhesus monkeys and chimpanzees is being further investigated.

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Zone Electrophoresis in Filter Paper of Serum I^{131} After Radioiodide Administration.* (19930)

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Recent observations in our laboratory(1) have shown that the serum of patients who have received large doses of radioiodide may contain variable quantities of an iodinated substance which is insoluble in butanol and from which thyroxine, diiodotyrosine, and iodide are liberated by alkaline hydrolysis. The data suggested that this substance might be thyroglobulin, or polypeptides resulting from breakdown of thyroglobulin, and the appearance of this material in the blood was related to the amount of radiation delivered to the normal or neoplastic thyroid tissue. Tong *et al.*(2) have made similar observations in rats. Several recent reports have described the behavior of the serum I^{131}, obtained after administration or radioiodide to patients, during zone electrophoresis of the serum in filter paper(3-5). The major portion of the serum organic I^{131} was found in a single area, having a mobility comparable to that of the alpha-globulins, although there was some variation in these reports as to whether the mobility was that of the $alpha_1$-globulins, the $alpha_2$-globulins, or intermediate between the two. A smaller portion of the radioactivity appeared in the albumin area. The patients used in these electrophoretic studies had received from 5 to 100 millicuries of radioiodide prior to the time the serum was examined. Insufficient data (*e.g.*, amount of I^{131} retained by the thyroid tissue, and estimated bulk of thyroid tissue) were given to permit evaluation of the destructive effect of the I^{131} doses. Although it is not likely that the amounts of I^{131} used in the patients with Graves' disease (5-20 millicuries) would have caused release of "non-thyroxine iodine" into the circulation, it is distinctly possible that the larger doses might have done so.

The following studies were undertaken to determine the electrophoretic mobility of the "non-thyroxine iodine" in serum following large doses of I^{131}.

Methods. Radioiodide (I^{131}) was obtained from Oak Ridge and administered orally, with 10 micrograms or less of carrier iodide, to 5 patients. Details with regard to diagnosis, amount of I^{131} administered, and estimated radiation to thyroid tissue are given in Table I. Measurement of I^{131} in the urine and calculation of the approximate radiation delivered to thyroid tissue during the second 24 hours were carried out as previously described(1). Serum was obtained from 4 to 9 days after the I^{131} was administered. In one patient (Case 1) serum was obtained 2 minutes after intravenous administration of 60 micrograms of I^{131}-labeled l-thyroxine.§ The serum was extracted with n-butanol, directly in some instances(1), and after precipitation of the serum proteins with trichloroacetic acid in others(2). When essentially all of the serum I^{131} is thyroxine, approximately 70 to 85% of the I^{131} is butanol-soluble by the direct method, and 90 to 100% is butanol-soluble by the trichloroacetic acid method. *Chromatographic analysis* was car-

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§ l-Thyroxine, obtained through the courtesy of Dr. John Mote of Smith, Kline & French Laboratories, Philadelphia, Pa., was labeled with I^{131} by Dr. G. Gleason of Abbott Laboratories, North Chicago, Ill. Its identity as thyroxine and its purity were confirmed in our laboratory by chromatographic analysis in the butanol-amyl alcohol-ammonia system, in 80 per cent phenol in water, and in butanol-acetic acid-H_2O (75:10:15)(6).

TABLE I. Summary of Data on 6 Patients and Serum Radioiodine Studies.

Case No.	Clinical data*	I^{131} dose (millicuries)	Approx. radiation to thyroid tissue in 2nd 24 hr (rep β)	Serum sample (days after dose)	BuOH solubility (% of total)	Chromatography (% of total in thyroxine band)	Fractional precipitation (% of total soluble in 1.75 M PO_4 buffer)	Iodide (% of total not precipitated with protein by 2.98 M PO_4 buffer)
1.	Athyreotic Angina pectoris (i.v. l-thyroxine)	0.5 (l-thyroxine)	—	(2 min.)	71† 96‡	93	—	—
2.	Graves'disease	20	5340	5	89‡	94	71	4
3.	Euthyroid Hypertensive heart disease Impaired renal function	9	5150	4	76‡	—	72	25
4.	Athyreotic Functioning thyroid carcinoma with bone metastases	150	3870	7	61†	80	74	3
5.	Athyreotic Functioning thyroid carcinoma with skull and soft tissue metastases	180	16500	6	38†	32	53	15
6.	Athyreotic Functioning thyroid carcinoma with bone and soft tissue metastases	207	15500	9	29‡	15	38	5

* Case 1 received a thyroidectomizing dose of I^{131} (50 millicuries) 6 mo prior to this study. Case 4 had had a total thyroidectomy followed by 2 large doses of I^{131} (>100 millicuries). The last dose was given 10 mo prior to this study. Case 5 received 247 millicuries of I^{131} 7 mo prior to this study. Case 6 received 175 millicuries of I^{131} 5 mo prior to this study.

† Direct method of extraction.

‡ Trichloroacetic acid method of extraction.

ried out by direct application of untreated serum to a narrow filter paper strip (Fisher E & A qualitative paper) and development in a system containing 70 parts n-butanol and 30 parts primary amyl alcohol, equilibrated with 2N ammonium hydroxide.‖ Approximately 50 μg of unlabeled thyroxine was streaked at the origin prior to application of the serum. Analysis for radioactivity and stable thyroxine were carried out as previously described(1). *Zone electrophoresis* in filter paper was performed by the methods of Kunkel and Tiselius(7) with slight modifications. The upper glass plate was raised about 0.5 cm above the paper and the sides were sealed with masking tape. The paper was pressed flat on the lower glass plate with a roller. The electrode vessels contained 1M KCl and were connected to the buffer vessels with agar-KCl bridges. Barbital buffer at pH 8.6, ionic strength 0.1, was used. Whatman 3 MM filter paper was cut into 3 cm by 30 cm strips, and two or, occasionally, three parallel strips were used for each run. Approximately 0.02 cc serum were applied to each strip. The potential across the platinum electrodes was 90 to 100 volts in 4 instances, and was 75 volts in Case 1 and 200 volts in Case 4. The current varied from 4 to 6 milliamperes among the individual runs and was maintained for 14 to 25 hours at room

‖ 70 cc n-butanol, 30 cc primary amyl alcohol and 100 cc 2N ammonium hydroxide were mixed and allowed to separate. The aqueous layer was placed in the bottom of a 15 cm x 45 cm covered glass cylinder. The alcohol layer was placed in a smaller vessel which fitted inside and on the bottom of the cylinder. After several hours were allowed for equilibration, the paper was suspended so that its end dipped into the alcohol mixture.

temperature. After staining of the paper by the technic of Durrum(8) (as modified by Kunkel and Tiselius) radioautographs were prepared by exposure to an x-ray film with a thin sheet of paper interposed, and/or the paper strip was cut into 0.5 cm segments and radioactivity measured with a thin mica window Geiger-Mueller tube. Since the strips were stained and washed prior to analysis for radioactivity, only protein-bound I^{131} was measured. The overall length of the serum patterns varied from 12 to 20 cm. In one instance (Case 6), electrophoresis was continued for 72 hours (length, 27.5 cm) in order to obtain more discrete protein bands.

Results. The butanol solubility and chromatographic analysis of the serum I^{131} recorded in Table I, indicate that in 4 instances (Cases 1, 2, 3, 4) most of the serum I^{131} (80% or more) behaved like thyroxine. In the remaining 2 patients (Cases 5, 6), a large proportion of the serum I^{131} (more than 60%) was insoluble in butanol and remained at the origin on the chromatographic strips. Representative chromatographic patterns are shown in Fig. 1.

Zone electrophoretic analysis of these serums, however, revealed similar patterns in all instances. Most of the radioiodine (approximately 85%) appeared in a single band which had a mobility intermediate between that of the $alpha_1$- and $alpha_2$-globulins, but overlapped both of these bands. The remainder of the radioiodine had a mobility similar to that of the serum albumin. Under the conditions used in these experiments, there was no demonstrable difference between the mobility of the butanol-soluble and the butanol-insoluble serum I^{131}, except that in one patient (Case 6) there was relatively more of the serum I^{131} (approximately 25%) in the albumin region. Representative electrophoretic patterns are shown in Fig. 2.

In order to test the possibility that these two forms of the serum I^{131} might be associated with a single protein, an attempt was made to separate them by fractional precipitation of the serum proteins. A modification of the method of Derrien, utilizing increasing concentrations of phosphate buffer, pH 6.5, was used(9-11). In Table I, the proportion of the serum I^{131} which was soluble

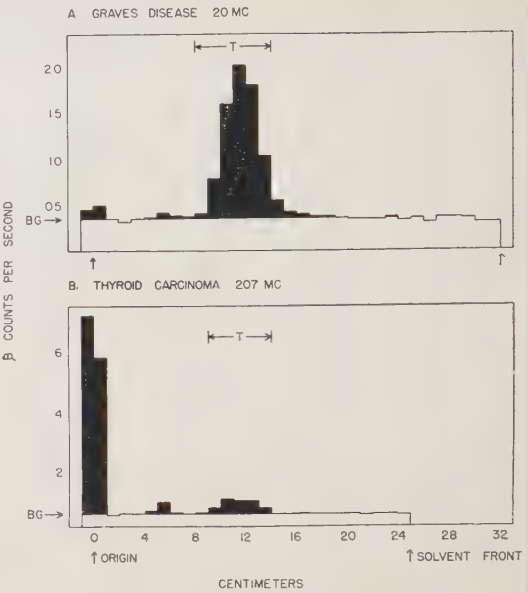

FIG. 1. Chromatographic analysis of untreated serum, butanol-amyl alcohol-ammonia system. A. Case #2. B. Case #6. The blackened area represents radioactivity in excess of background (BG). "T" indicates the position of stable thyroxine. The unlabeled band at approximately Rf 0.2 is in the position of inorganic iodide.

at a phosphate buffer concentration of 1.75 Molar¶ is recorded. It may be seen that when the serum I^{131} was largely butanol-soluble, more than 70% of the radioiodine remained in solution in 1.75 Molar phosphate buffer (Cases 2, 3, 4); whereas relatively smaller amounts of radioiodine were soluble at this salt concentration when butanol-insoluble I^{131} was present in the serum (Cases 5, 6). The greater the proportion of butanol-insoluble I^{131} in the serum, the more I^{131} was recovered in the phosphate buffer precipitate. When the protein precipitated by 1.75 Molar phosphate buffer in Case 6 was redissolved in distilled water and subjected to zone electrophoresis, the I^{131} was found to have the same mobility as in whole serum, although little if any of the alpha globulins of the serum was present.

¶ 1.75 Molar phosphate buffer concentration was attained by adding an equal volume of a stock solution (an equimolecular mixture of K_2HPO_4 and KH_2PO_4, 3.5 Molar), to serum diluted with water so that the final serum dilution was 1:30. This was, therefore, a 50% solution of the stock solution.

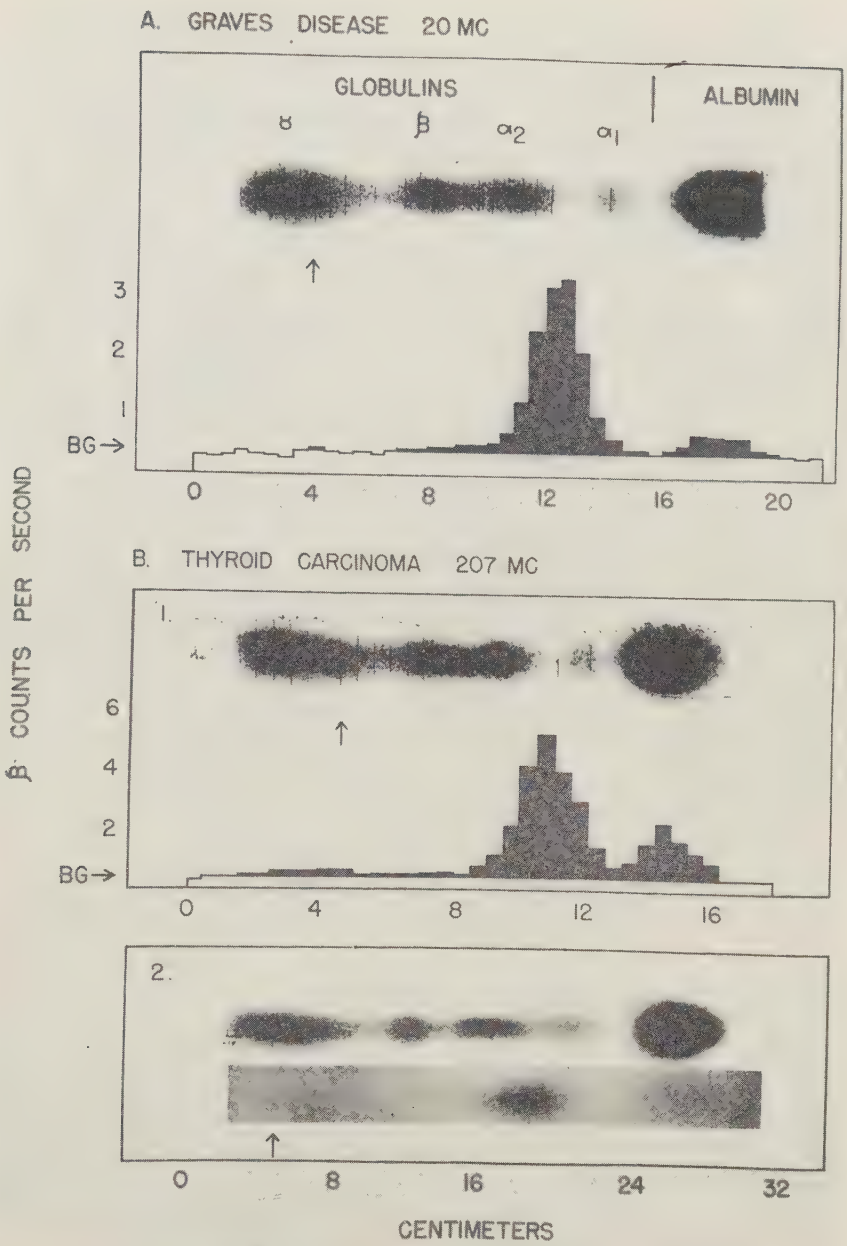

FIG. 2 Photographs of electrophoretic patterns of the serum proteins from Case #2 (A), and Case #6 (B) are compared with the location of radioactivity determined by counting 0.5 cm segments of the strips (A, B1) and by radioautography (B2). The arrows indicate the positions at which the serum samples were placed on the paper. The blackened areas in graphs A and B1 represent radioactivity in excess of background (BG). The small amount of radioactivity apparent in the gamma globulin area in B1 was found in no other instance. The radioautograph in B2 is intended to demonstrate the location of the major band of radioactivity in the alpha globulin region. The gray areas on the remainder of the radioautograph are due to technical problems in its reproduction and not to radioactivity, thus obscuring the band in the albumin region.

This method of fractional precipitation of the serum proteins also enabled estimation of the radioiodide content of the serum by determination of the I^{131} which was not pre-

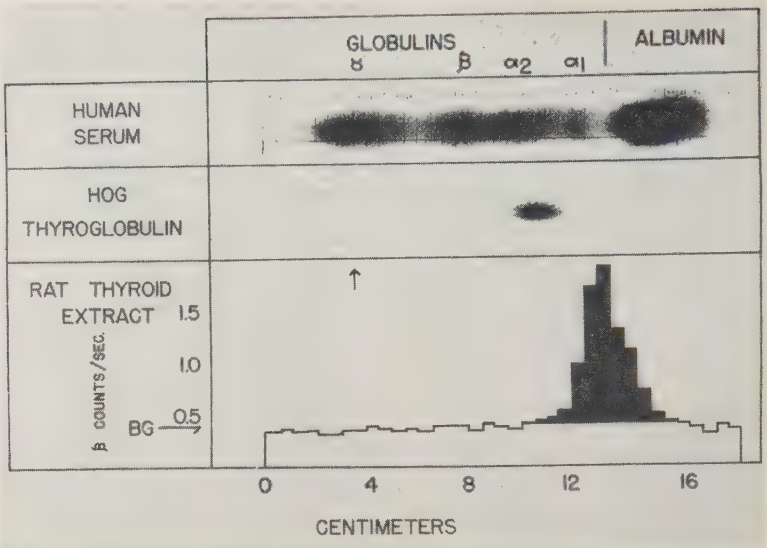

FIG. 3. Photographs of electrophoretic patterns of: A. Normal human serum containing rat thyroid extract, 1 part per 100, and B. Purified hog thyroglobulin. The graph (C) represents the location of the radioactivity in A due to the rat thyroid extract, as determined by counting 0.5 cm segments of the strip. The blackened area in C represents counts above background (BG). In B, a small amount of protein tailing from the origin (arrow) to the major band is not visible in photograph.

cipitated with the serum proteins by 2.98 Molar phosphate buffer(11).** These values are recorded in Table I.

In order to determine whether thyroglobulin might have an electrophoretic mobility comparable to that of the serum I^{131}, thyroglobulin from two animal species was studied as follows:

1. Purified hog thyroglobulin†† was obtained in lyophylized form, containing approximately 25% NaCl. A 1% solution in distilled water was prepared and immediately subjected to zone electrophoresis. Almost all of the protein moved in a single band with a mobility intermediate between $alpha_1$- and $alpha_2$-globulin, as demonstrated by a parallel run of normal human serum (Fig. 3)

2. A crude extract was prepared from the thyroid gland of a rat, which had received 60 microcuries of radioiodide intraperitoneally 48 hours earlier, by grinding the thyroid with 0.5 cc of isotonic saline at 0°C and allowing the larger particles to sediment. Within 15 minutes after the animal was killed, an 0.01 cc aliquot of the extract was subjected to zone electrophoretic analysis. Parallel to this, was a run containing 0.02 cc of a mixture made by adding 0.05 cc of the thyroid extract to 5 cc of normal human serum. The results indicated that the I^{131} in the rat thyroid extract had a mobility intermediate between that of the $alpha_1$-globulin and the albumin in the serum. The mobility of the I^{131} in the thyroid extract-serum mixture is shown in Fig. 3. In the absence of serum, approximately 60% of the I^{131} had an identical mobility, the remainder tailing from this band to the point of origin.

Discussion. These results coincide with the findings of others with regard to the zone electrophoretic pattern of the normal serum I^{131}. It appears that thyroxine is attached to a protein (or proteins) which moves with the alpha-globulins at pH 8.6 but which differs from those making up the bulk of the $alpha_1$-

** 85% by volume of the phosphate buffer stock solution.

†† Purified hog thyroglobulin was obtained through the courtesy of Dr. Robert Kroc, Chilcott Laboratories, Morris Plains, N. J. (Lot No. 91921). It was prepared from hog thyroid extract by precipitation with ammonium sulfate at about pH 6. Ultracentrifuge analysis in the laboratory of Dr. Mary L. Peterman, Sloan-Kettering Institute, showed it to be 75% homogeneous with a sedimentation constant of 17.2 S in a 1% solution.

and $alpha_2$-globulins. Presumably, this protein is present in quite small amounts, and it has a peculiar affinity for thyroxine, since it contains practically all of the thyroxine added to normal serum *in vitro*(3) or administered to a patient by intravenous injection. It is of interest that no difference in electrophoretic mobility was detected between the serum I^{131} in a patient with a normal thyroid gland, a patient with Graves' disease, and a patient with functioning thyroid carcinoma and no normal thyroid tissue.

It is clear, however, that the behavior of serum iodine during electrophoresis at pH 8.6 cannot be used as evidence concerning the identity of the normally circulating thyroid hormone, since the normal, butanol-soluble serum I^{131}, and the butanol-insoluble serum I^{131}, ("non-thyroxine I^{131}") that follows thyroid tissue destruction with radioiodine, have essentially the same mobility. It is possible that under conditions of pH and ionic strength other than those used in this study, a differentiation might be obtained.

The serum I^{131} which appears in the albumin band may be an artifact since there is some evidence that serum albumin is capable of binding thyroxine *in vitro*(12), and it is possible that, during handling of the serum, some of the thyroxine may be released from its specific protein. The finding of relatively more I^{131} in the albumin band in Case 6 may, however, be of significance.

The ability to differentiate between the butanol-soluble serum I^{131} and the butanol-insoluble serum I^{131} by fractional precipitation with phosphate buffer is evidence that the normal "thyroxine-binding protein" in the serum is not identical with a protein released from irradiated thyroid tissue (*e.g.*, that it might be non-iodinated thyroglobulin). This finding also indicates that the radiation-induced "non-thyroxine iodine" in the serum is probably not attached to the normal "thyroxine-binding protein."

Considerations presented elsewhere(1,2) suggest that the "non-thyroxine iodine" may be thyroglobulin. While the identity in the mobility in zone electrophoresis of "non-thyroxine I^{131}" and purified hog thyroglobulin is consistent with this view, it cannot be considered proof, especially in view of the foregoing discussion. The difference in mobility between the purified hog thyroglobulin and the rat thyroid extract may be due to a species difference or to an effect of other substances in the crude thyroid extract. It may be noted that studies with purified hog and beef thyroglobulin in classical boundary electrophoresis systems(13,14) also indicate that this substance has a mobility of the order of that of the alpha globulins of human serum. This is true at pH 7.7 as well as at pH 8.3.

It is still possible, nevertheless, that some or all of the thyroglobulin released from the thyroid tissue may have been broken down into smaller components.

Summary and conclusions. 1. Serum from 6 patients who had received radioiodide or I^{131}-labeled thyroxine was studied by zone electrophoresis in filter paper, at pH 8.6. Most of the I^{131} had a mobility intermediate between the $alpha_1$- and $alpha_2$-globulins, and a small proportion had the mobility of albumin. No differences were detected between thyroxine added to serum (*in vivo*) and the thyroid hormone secreted by a normal thyroid gland, by a Graves' disease thyroid gland, and by a functioning thyroid carcinoma. 2. Two patients had received very large doses of radioiodide and their serum contained large amounts of butanol-insoluble I^{131}, whereas in the other patients the serum I^{131} was largely butanol soluble, and behaved like thyroxine on paper chromatography. These two forms of serum I^{131} had similar mobility on zone electrophoresis, but could be differentiated by fractional precipitation of the serum proteins with phosphate buffer. The radioiodine, therefore, was probably attached, in each instance, to a different protein, and the identity in their mobilities was fortuitous. 3. Zone electrophoresis of purified hog thyroglobulin, and of I^{131}-labeled rat thyroglobulin in a crude thyroid extract, revealed that these substances also had a mobility in the range of the alpha globulins of human serum. This finding is compatible with the previous suggestion that the butanol-insoluble I^{131} ("non-thyroxine I^{131}") may be thyroglobulin, released into the circulation from damaged thyroid tissue.

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Availability of Vitamin E in the Newborn Infant.* (19931)

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The erythrocytes of rats deficient in vit. E may be hemolyzed by dialuric acid(1,2) *in vivo* and *in vitro*. They are also susceptible to the hemolyzing effect of dilute solutions of hydrogen peroxide(3). Vit. E given *in vivo* or added to suspensions of red cells *in vitro* prevents the hemolysis by dialuric acid as well as by hydrogen peroxide.

The hemolytic action of hydrogen peroxide differs in several respects from that of dialuric acid. Hemolysis of cells deficient in vit. E is complete in 15 to 30 minutes with dialuric acid, while with hydrogen peroxide hemolysis progresses only slowly and even after prolonged incubation is usually less marked than with dialuric acid. Moreover, the red blood cells of newborn infants show increased susceptibility to hydrogen peroxide and at the same time complete resistance to dialuric acid. In adults hemolysis of red blood cells by hydrogen peroxide rarely exceeds 5%(3).

The question arose whether the hemolysis in the blood of newborn infants by hydrogen peroxide might be related to vitamin E deficiency and whether addition of vit. E either in the test tube to a suspension of red blood cells, or through ingestion by the newborn infant, or, prenatally, by the pregnant mother, would restore the normal resistance of the erythrocytes toward hydrogen peroxide.

Experimental. Observations were made on normal newborn infants in the Nursery of the Hospital of the University of Pennsylvania. Normal adults served as negative controls while vit. E deficient rats were used as positive controls. *Blood* was collected in 0.9% NaCl-1% sodium citrate solution (about 15 drops of blood in 2 ml) in a 15 ml centrifuge tube. The suspension was centrifuged, the supernatant fluid withdrawn, and the cells made up in a 2.5% suspension (estimating the volume of cells from the graduations on the tube) with a mixture of equal parts of normal saline and phosphate buffer, pH 7.4.† After incubation at 37°C for one hour the tubes were again centrifuged, the supernatant fluid withdrawn, and a 5% suspension of the cells in normal saline made.

* Preliminary report given at Meeting of the Am. Pediat. Soc., Atlantic City 1951, see *Am. J. Dis. Child.,* 1951, v82, 237.

† Phosphate buffer, pH 7.4:

	ml
Potassium monophosphate, 0.2 M	25.0
Sodium hydroxide, 0.2 M	19.7
Water to make	100.0

Into 3 centrifuge tubes were measured 0.25 ml cell suspension, 0.20 ml phosphate buffer and 0.05 ml 12% hydrogen peroxide (1 volume of 30% H_2O_2 + 1.5 volumes phosphate buffer). A fourth tube containing no peroxide (0.25 ml phosphate buffer) served as a negative control. All tubes were incubated at 37° for 15 minutes and allowed to stand at room temperature for 2¾ hours. To the negative control and to 2 of the tubes containing peroxide was added 4.5 ml saline-buffer solution. The tubes were mixed by inversion and centrifuged. The color of the supernatant liquid was measured in a Klett-Summerson photometer using the No. 54 filter. Four and one half ml of distilled water was added to the remaining tube to cause complete hemolysis of the cells and the color read in the photometer. Better checks, between duplicate determinations, may be obtained by using the peroxide in more dilute solution, 0.25 ml of 2.4% H_2O_2 instead of 0.05 ml of 12% H_2O_2, as suggested by Gordon and de Metry(4). The percent of hemolysis caused by hydrogen peroxide was calculated from the ratio of the average reading of the tubes with saline-buffer and that of the completely hemolyzed tube, both readings being corrected by subtracting the reading of the negative control. In studies in which the effect of tocopherol *in vitro* was tested the desired amount of tocopherol was added before the first incubation.

Results. The hemolysis test with hydrogen peroxide was carried out first on normal infants on the 1st, 2nd, and 5th days after birth. Neither the mothers nor the infants received supplements of vit. E. The results together with positive and negative controls are summarized in Table I.

In a further group of 17 newborn infants the hydrogen peroxide test was carried out before and after addition of tocopherol to a suspension of red cells. The degree of hemolysis before treatment was found to be 36 ± 4.5% which was reduced after addition of tocopherol to the red blood cell suspension(3) to 0.6 ± 0.4%, indicating complete and regular response.

The effect of tocopherol on hemolysis by hydrogen peroxide was demonstrable also when the tocopherol was given by mouth to

TABLE I. Hemolysis of Red Cells by Hydrogen Peroxide in Normal Untreated Newborn Infants.

Type of cells	No. of determinations	Hemolysis (%)
Newborn infant, 1st day	30	27 ± 3.9
2nd	30	31 ± 4.3
5th	28	16 ± 4.4
Human adult	75	3 ± .6
Rat (vit. E deficient)	77	79 ± 1.6
Rat (treated with vit. E)	33	9 ± 2.0

TABLE II. Hemolysis of Red Blood Cells by Hydrogen Peroxide in Normal Infants Born of Mothers Receiving Prenatal Vitamin E Medication.

Type of cells	No. of determinations	Hemolysis (%)
Newborn infants, mothers receiving Vit. E (150 mg daily)		
1st day	16	22 ± 4.3
2nd	16	25 ± 3.3
5th	12	6 ± 2.5
Newborn infants, mothers receiving No vit. E		
1st day	15	25 ± 5.5
2nd	15	36 ± 5.9
5th	12	14 ± 6.9
Normal adults	41	2 ± .2
Rats (E-deficient)	39	82 ± 2.0

the newborn infants. In 11 normal infants hemolysis on the first day was 42 ± 6.1%. After feeding of 50 mg of water-miscible mixed tocopherols (Esorb, Wyeth) the hemolysis fell next day to 6 ± 2% and on the 5th day to 3 ± 1%.

Similar results were previously recorded in rats(5,6). The blood of newborn rats is, as a rule, largely hemolyzed by dialuric acid as well as by hydrogen peroxide. Tocopherol not only in the test tube but also *in vivo* through diaplacental transfer of tocopherol given to the pregnant animal (5 mg daily) prevents hemolysis. We have studied the response to tocopherol through attempted diaplacental transfer in two series of observations on newborn infants. In the first group we gave the mother by mouth during the last 2 to 4 weeks of pregnancy 150 mg of water miscible mixed tocopherols (Esorb, Wyeth) daily. The results are summarized in Table II. There was no difference in the degree of hemolysis of the two groups on the first day, and the reduction of hemolysis on the 5th day in the group of newborn infants born of mothers receiving supplements of vit. E was

not statistically different from that of the control group.

In a second smaller series of observations the mothers were given 500 mg of mixed tocopherols daily during the last 2 to 4 weeks of pregnancy. Even with this larger dose of tocopherol the "prophylactic" approach proved to be ineffective. In the treated group, comprising 10 mothers, the rates of hemolysis in the newborn infants showed the following average figures: 1st day, 47 ± 11; 2nd day, 24 ± 5; 5th day, 29 ± 9.

In the control group (8 mothers without treatment) the corresponding figures were: 1st day, 70 ± 6; 2nd day, 47 ± 9; 5th day, 27 ± 7.

Thus, in newborn infants, the diaplacental protection observed in rats was not achieved even when the mother was given very high doses of vitamin E. This result is in contrast to the prompt effect of tocopherol on hemolysis of erythrocytes by hydrogen peroxide when given directly to the newborn human infant by mouth.

Discussion. The susceptibility of the red blood cells of newborn infants to dilute solutions of hydrogen peroxide is analogous to the susceptibility of erythrocytes of newborn rats to hydrogen peroxide as well as to dialuric acid. Since vitamin E *in vitro* and *in vivo* restores normal resistance of the red blood cells, the conclusion appears to be warranted that newborn rats and newborn infants suffer from "physiological" vit. E deficiency. Gordon and de Metry confirmed these observations on premature infants(4). Furthermore, the analytical data of Moyer(7) and of Wright, Filer and Mason(8) are in good accord with the results of the hemolysis tests.

The hemolysis test has the advantage of measuring the biologically active portion of total tocopherol(9). The protein bound tocopherol is unavailable for the hemolysis test (2).

The positive hemolysis test in newborn rats may be reversed not only through direct contact of vit. E with the erythrocytes but also by giving vit. E to the pregnant mother. The placental barrier is apparently less easily traversed in case of the human fetus. In the statistical average no definitely positive results were obtained even after daily doses of 500 mg of mixed tocopherols given the mother during the last few weeks of pregnancy. In contrast direct medication was promptly effective in the newborn infant. On the basis of observations made in rats, prophylactic medication of vit. E was proposed(5) via the pregnant mother in conditions such as Rh incompatibility or in diabetes. In the light of the present findings it appears that such approach might not be successful unless the amount of vit. E required to prevent hemolysis in diabetic pregnancy(5,10), or hepatic injury(5) and edema of the placenta(11,12) in Rh incompatibility is less than the level necessary for reversal of the positive hemolysis test with hydrogen peroxide. No explanation can be given for the very high threshold of human placenta for vit. E.

Summary. Red blood cells of newborn infants are hemolyzed by dilute solutions of hydrogen peroxide. This hemolysis may be prevented by vit. E *in vitro* or *in vivo*. However, even with high daily doses of vit. E (500 mg of mixed tocopherols) given the mother during the last weeks of pregnancy the hemolysis test in the newborn remained unaltered.

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Estrogenic Effect of Tri-p-anisylchloroethylene (TACE)* in the Human Postmenopausal Female. (19932)

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The study reported here was undertaken to determine the size of the minimal effective dose of Tri-p-anisylcholoroethylene (TACE)* in postmenopausal human subjects. TACE is a synthetic estrogen reported to have a remarkably prolonged effect after oral administration to laboratory animals (rats and monkeys) (1,2); although it has been used therapeutically in human patients(3) almost no reports are available regarding its estrogenic potency in the human subject, and the only comparisons that have been made between it and other estrogens have been done on laboratory animals.

Methods and materials. The subjects were 19 postmenopausal human females, inmates of a county home for the aged. Their ages ranged from 50 to 85 years and the postmenopausal interval, from 3 to 45 years. Each subject included in this study was given a gynecologic examination and judged to be free of pelvic disease. The criterion of estrogenic effect used was the degree of growth of the vaginal mucosa, as judged by the changes in the vaginal smear. All smears were obtained with the subjects in lithotomy position, using a dry cotton tipped swab inserted as deeply into the vagina as possible in the direction of the posterior fornix. On being removed from the vagina, the swab was rolled out on a clean glass slide, and the latter immediately dropped into the fixative solution (equal parts of 95% ethyl alcohol and ethyl ether). The smears were stained either the same or the next day with Schorr's single differential stain(4), and mounted in balsam. The entire area of each smear was examined under low power, and the approximate percentage of cornified, intermediate and basal epithelial cells, leucocytes, debris, and cytolysis estimated, without actually making cell counts of the smears. They were then given an arbitrary grade according to the following scheme: 0—primarily basal cells, with or without leucocytes; +—very few basal cells, with the predominant cells derived from the intermediate cell layers; ++—mostly intermediate cell types, with less than 50% of the cells cornified; +++—same as plus 2, except that at least 50% of the cells were cornified, and the remainder late intermediate cell types. All smears were graded without knowledge of the particular subject or treatment involved. Each subject had a typical postmenopausal smear at the start of the study (grade of 0, according to the above scheme); if she served for more than one test dose, the smear was allowed to return to the pretreatment level before the second or other treatments were started.

Two total doses of TACE were tried: 24.0 and 48.0 mg. The 24.0 mg dose was administered either as a single dose, or as a divided dose of 6.0 mg given on each of 4 consecutive, or on each of 4 alternate days, in either capsule or tablet form. The 48.0 mg dose was given as four 12.0 mg doses on each of four consecutive days, in either tablet or capsule form. Smears were taken before treatment, approximately every other day during the course of, or in the first week following treatment, and then at intervals varying from 3 times to once per week until complete regression was seen.

Results. The results are presented in Table I. It can be seen that 24.0 mg as a single dose is not sufficient to bring about more than a moderate amount of vaginal growth but that the effectiveness of this dose is greatly increased when it is administered as a divided dose on 4 consecutive days; the effect is possibly somewhat less than that produced by 48.0 mg administered in a similar manner. The data also indicate the possibility that the capsule may be a more effective method of

* TACE is the trade-mark of The Wm. S. Merrell Co., Cincinnati, for its brand of chlorotrianisene (tri-p-anisylchloroethylene).

TABLE I. Effect of TACE on Cornification of the Postmenopausal Human Vagina.

Total dose TACE admin., mg	Method of admin.	Total No. subjects	Maximal effect*		Onset of max effect (days) †	Duration of any effect (days) †
			Subjects	Grade		
24	Single dose					
	Capsules	9	6	+	6-10	10-13
			3	++		
	Tablets	8	7	+	6-10	10-13
			1	++		
24	6 mg on each of 4 consecutive days					
	Capsules	9	1	0	5-6	10-14
			1	+		
			4	++		
			3	+++		
	Tablets	5	2	++	5-6	10-14
			3	+++		
24	6 mg on each of 4 alternate days‡	5	2	+	1-2	10-12
			2	++		
			1	+++		
48	12 mg on each of 4 consec. days§	6	1	+	2-6	17
			1	++		
			4	+++		

* See text for description of grades.
† From the time of administration of the last dose.
‡ Administered in capsule form only.
§ Three of these patients received this dose in capsule, and three in tablet form. The differences in effect were too small to justify their presentation as separate groups.

administration than the tablet form but until further studies on a larger number of patients are completed, this must remain in the realm of conjecture.

Since the study was undertaken to determine the minimal effective dose of TACE, prolongation of the estrogenic effect which occurs at higher dosage levels(5) was not expected. Our data show that the duration of maximal effect of these doses of TACE was in the neighborhood of about a week from the time of administration of the last dose, and that a period of roughly 2 to 6 days elapsed after administration of the last dose before the maximal effect was seen. A small comparative study of 2 other oral estrogens (ethinyl estradiol and dienestrol) was run. The ethinyl estradiol was administered as six daily doses of 0.05 mg each, and the dienestrol as 6 daily doses of 0.10 mg each. Both of these induced approximately the same amount of vaginal growth as did 24.0 mg of TACE administered as a divided dose on each of 4 consecutive days. The time of onset of the maximal effect, and the duration of effect with both of these was also roughly equivalent to this dose of TACE.

Conclusions. The minimal effective dose of TACE after oral administration required for virtually complete cornification of the postmenopausal human female vagina is in the neighborhood of 24 to 48 mg administered as 4 daily doses of 6 to 12 mg each. Similar amounts of TACE administered on alternate days do not induce much cornification of the vaginal mucosa, but do lead to some growth of the intermediate cell layers. The duration of effect of these minimal effective doses of TACE was not remarkably different from that of other oral estrogens (ethinyl estradiol, dienestrol), but it is possible that its prolonged effect appears only with doses greater than the minimal ones.

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Effect of Leukocyte-Promoting Factor (LPF) on Survival of X-Irradiated Mice.* (19933)

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The beneficial effect of spleen shielding on survival of X-irradiated mice has led to the hypothesis that a "humoral" substance is produced by the protected spleen, and that this substance stimulates rapid recovery of the hematopoietic tissues(1). Implantation of normal spleens in irradiated mice following radiation exposure is also reported to increase survival. The therapeutic effect of the implantation is considered additional evidence of a "humoral" mechanism of protection(1). As yet the only cell-free factor that has been isolated from biological material which might produce this protective effect following radiation is the factor described by Menkin which induces release of leukocytes into the peripheral blood and produces hyperplasia of granulocytes and megakaryocytes in the bone marrow(2,3).

The present study was undertaken to determine whether or not Menkin's leukocytosis-promoting factor (LPF) when given therapeutically would increase survival in irradiated mice.

Methods. A total of 200 female white Swiss mice 8 to 10 weeks of age were used in the present study. Prior to treatment the mice were randomly distributed into treatment groups by a random number table. Prior to and following treatment the mice were kept 10 to a cage and given water and Purina laboratory chow *ad libitum*. The leukocytosis-promoting factor used was freshly prepared by V. Menkin from a sterile pleural exudate induced in a dog. The material obtained was a fluffy white powder. It was stored in a desiccator *in vacuo* over phosphoric anhydride. Fresh solutions were made up for each injection by dissolving a suitable amount of the LPF in sterile saline. With the exception of some of the injections in the preliminary portion of the study which were done intravenously into the tail vein, all injections were made intraperitoneally. Injection volumes varied from 0.1 to 0.5 cc. Blood counts were performed in some of the mice by cutting of the tip of the tail to obtain freely flowing blood which was then aspirated into standard white cell pipettes. Counts were made in standard hemocytometer chambers. *X-rays* were delivered from a Picker industrial model machine operated at 250 KVP and 15 ma. The other factors were: added filtration of ¼ mm Cu and 1 mm Al, 55 cm tube-specimen distance, dose rate—70 r/min. The mice were exposed 20 at a time in shallow lucite cages curved on a radius of 55 cm to minimize differences in radiation intensity between the center and periphery of the cage.

Results. In preliminary studies a dose of LPF was established which would produce maximal leukocytosis. Eight groups of 5 mice each were injected either intravenously or intraperitoneally with various doses of LPF. Four hours later white cell counts were performed and the degree of leukocytosis determined. These data are given in Table I, and they show that a dose of 50 mg/kg produces maximal leukocytosis at 4 hours and that intraperitoneal injections produce as great a rise as intravenous injections. For these reasons 50 mg/kg given intraperitoneally were used in the study on therapy.

Four groups of 40 mice each were irradiated with 450, 500, 600, and 700 roentgens of X-rays, respectively. After irradiation, 10 mice from each dosage group were set aside for treatment with LPF, and the remaining 30 in each group were used as controls. Beginning the day of radiation and continuing for the following 5 days the mice were injected with 50 mg LPF/kg intraperitoneally. The control mice were injected similarly with saline. Survival was then followed for 30 days.

Table II shows the 30-day survival of the various groups. It can be seen that treatment with LPF did not significantly increase sur-

* Work done under the auspices of the AEC.

TABLE I. Degree of Leukocytosis Produced in Mice at 4 Hours by Various Doses and Routes of Administration of LFP. 5 mice in each exp.

Route of administration	Dose LPF (mg/kg)	Avg change in white cell count at 4 hr (% of pre-inj. count)
Intraperitoneal	5	110
	10	119
	50	153
	100	119
	*	96
Intravenous	10	105
	50	156
	*	107

* Saline control.

TABLE II. 30-Day Survival of Irradiated Mice Treated with LPF or Saline Intraperitoneally.

No. mice	Dose of X-rays (roentgens)	Treatment	30-day survival	% survival
10	450	50 mg LPF/kg for 6 days	9	90
	500		4	40
	600		2	20
	700		0	0
30	450	Saline inj. controls	22	73
	500		13	43
	600		2	7
	700		0	0

vival. Analysis of the data by the probit analysis method described by Finney(4) gives an LD_{50}^{30} value of 489 r for the control mice and 518 r for the mice injected with LPF. These differences are not considered significant.

Discussion. Treatment of irradiated mice with leukocytosis-promoting factor did not significantly increase their 30-day survival. This fact strongly suggests that if survival of irradiated mice into which normal spleens have been implanted is due to a "humoral" factor released by the spleen, such a factor is unrelated to the specific leukocyte-promoting factor obtained from sterile exudates.

Summary. The present study does not contribute to the discussion as to whether or not a "humoral" substance is secreted by the spleen. It does show, however, that LPF, a logical therapeutic agent, is ineffective in reducing X-ray mortality in mice.

We are indebted to Dr. Valy Menkin for providing us with a potent sample of LPF and for helpful suggestions as to the plan of the experiment.

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Mechanism of Renal Glycosuria in ACTH-Treated Premature Infants.* (19934)

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In a group of 7 premature infants given ACTH in the treatment of early retrolental fibroplasia, 5 developed glycosuria, and in none of these was there an associated hyperglycemia. These data implied a glomerulotubular functional imbalance in the handling of glucose. Dustan *et al.*(1) concluded that glycosuria during cortisone and ACTH administration results from a depression of the glucose Tm. However, Earle *et al.*(2) concluded from their studies that there was no consistent decrease in the maximum ability (Tm) of the tubules to reabsorb glucose under the influence of ACTH and believed that the glycosuria results from a rise in glomerular filtration rate producing an increased glucose

* Aided by a grant from Mead Johnson and Co.

TABLE I. Effect of ACTH on Renal Function.

	Name & date	Age, days	Wt, g	Therapy	GFR, ml/ min./1.73 M²	TmG, mg/ min./1.73 M²	GFR / TmG	S.A.
				Patients with glycosuria				
1.	W 5/13/51	12	1650	Prior to ACTH	43	93	.47	.144
					41	88	.46	
	W 6/14	33	1955	ACTH for 21 days	27	15	1.80	.161
					26	14	1.80	
	W 6/30	49	2240	Post ACTH period	70	177	.40	.177
					70	169	.42	
2.	JL 7/25/52	26	1670	Prior to ACTH	22	48	.46	.145
					25	48	.52	
	JL 8/ 8/51	39	1690	ACTH for 13 days	43	53	.81	.146
					41	55	.75	
3.	JL 7/26	27	2000	Prior to ACTH	43	86	.50	.163
					44	98	.45	
	JL 8/10	42	2080	ACTH for 10 days	43	51	.85	.168
					41	42	.97	
	JL 8/22	54	2160	ACTH for 22 days	67	74	.91	.172
					55	67	.82	
4.	P 6/21	54	1820	ACTH for 17 days	61	92	.67	.154
					59	89	.66	
5.	M 1/30/52	87	2750	ACTH for 21 days	68	92	.74	.202
					64	83	.76	
				Patients without glycosuria				
6.	S 11/ 1/51	37	2000	Prior to ACTH	31	60	.51	.163
					36	68	.53	
	S 11/30	67	2830	ACTH for 21 days	46	89	.52	.206
					41	83	.49	
7.	N 12/ 5	41	2270	Prior to ACTH	34	75	.47	.177
					34	72	.50	
	N 12/12	47	2320	ACTH for 6 days	31	71	.43	.180
					29	75	.49	

load which is above the capacity of the normal tubules to reabsorb. Because of these conflicting views, and in order to determine the mechanism involved in the glycosuria of these premature infants, the following study was undertaken.

Materials and methods. Premature infants weighing between 1650 and 2750 g, and between 12 to 87 days of age were studied. Glomerular filtration rate and glucose Tm were estimated before, during, and after a period of ACTH therapy. The glomerular filtration rate was measured by inulin clearance. The inulin was administered subcutaneously as a 10% solution 90 minutes before estimating the glomerular filtration rate. Inulin was determined by the method of Hubbard and Loomis, using the acid mixture of Harrison(3). To obtain the glucose Tm a 15 to 20% glucose solution was infused intravenously at a rate of about 1 ml/min. (200 mg glucose/min.). Forty-five minutes were allowed for equilibration of the glucose. Capillary blood glucose values of 450 to 1020 mg % were obtained. In each case the load/TmG ratio was over 1.30. Glucose was determined by the method of Nelson(4). Both glomerular filtration rate and glucose Tm were determined within 3 to 4 hours after the last injection of ACTH. ACTH was given intramuscularly in a dosage of 40 mg daily for the first 2 weeks, then reduced to 20 mg daily for the third week, and finally to 15 mg daily for the fourth week. The total daily dosage was divided into 4 equal parts and given at 6-hour intervals. Since varying time elapsed in these patients between the control and the experimental periods, it became important to estimate the normal maturational change in glomerular filtration rate and glucose Tm occur-

TABLE II. Glucose TmG in Premature Infants.

Name & date	Age, days	Wt, g	Therapy	GFR, ml/ min./1.73 M^2	TmG, mg/ min./1.73 M^2	GFR / TmG	S.A.
8. M 10/ 4/51	27	1930	None	42	72	.59	.160
				48	82	.58	
M 10/17	41	2250	"	54	119	.46	.177
				44	125	.35	
9. E 9/13	30	2290	"	39	85	.46	.179
				36	73	.50	
E 9/20	47	2550	"	46	101	.46	.192
				46	110	.42	
				46	100	.46	
10. St. 7/12	7	1390	"	29	50	.58	.126
				30	47	.63	
11. L. 7/25	26	1670	"	22	48	.46	.145
				25	48	.52	
12. JL 7/26	27	2000	"	43	86	.50	.163
				44	98	.45	

ring over this period of time. For this reason a group of 5 premature infants were studied to determine the effects of aging alone on these functions over a similar period of time.

Results. Table I shows the data on a group of 5 premature infants who developed glycosuria under ACTH therapy, and 2 who did not develop glycosuria. The effect of ACTH on glomerular and tubular functions in these maturing infants can be gauged only by comparison with the changes in glomerular filtration rate and glucose Tm naturally occurring with aging. Table II shows the effect of aging alone on these two functions.

It is apparent from Table II that during the length of time involved in the ACTH study (13 to 37 days) there is but small increase in glomerular filtration rate, but a somewhat more significant rise in glucose Tm as a result of growth. In the infants receiving ACTH, a rise in glomerular filtration rate over the expected increase occurred in 3 of the 5 infants. In one case it was apparent on the 13th day of ACTH administration; in another it occurred only after more prolonged ACTH effect (27 days), and the rise was slight in the third case after 21 days. In case I there was a fall in glomerular filtration rate. This patient developed an associated albuminuria and hematuria. In the fifth child the glomerular filtration rate was essentially unchanged. In the 5 infants where glucose Tm was compared before and after ACTH, there was a fall in 2, 2 remained unchanged, and in the fifth child there was a rise. The rise in glucose Tm in this latter patient treated for a 30-day period approximately equals the rise found in the 2 normal infants over a shorter period of time as a result of normal growth. In the 2 instances where the glucose Tm was unchanged with ACTH, we would have anticipated over this period of time a rise comparable to the two control cases (25% to 50%).

The imbalance of glomerulo-tubular function for glucose is strikingly demonstrated by comparing the GFR/TmG ratio in the ACTH-treated infants with the preACTH period and the control non-ACTH group. In the non-ACTH infants the ratio varies between 0.35 to 0.59. This is in agreement with values obtained in premature infants by Tudvad(5). This author also demonstrated a fall in this ratio with aging. In the ACTH-treated infants with glycosuria the ratio ranged from 0.66 to 1.80. This rise in ratio cannot be accounted for by increasing maturity. In infants 4 and 5, where no preACTH period was obtained, the high GFR/TmG ratios indicate the ACTH effect.

In 2 infants where glycosuria was not induced by the ACTH the ratio of GFR/TmG was not elevated. There was also no depression of the glucose Tm in these 2 infants. In the case where glomerular filtration rate was increased the glucose Tm was increased pro-

portionately, keeping the GFR/TmG ratio unchanged. The reason for the lack of ACTH effect in these 2 children is not apparent. However, in case 7 the lack of response may be related to the short period of ACTH administration, (6 days).

Conclusions. Glycosuria resulting from ACTH administration in premature infants is due to an imbalance of glomerulotubular function demonstrated by a rise in GFR/TmG ratio. The cause of the rise in this ratio varied and was due either to a disproportionate rise in glomerular filtration rate or fall in glucose Tm. The nonglycosuric ACTH-treated infants did not show this rise in the GFR/TmG ratio.

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A Glass Wool Matrix for Roller Tube Tissue Cultures.* (19935)

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The isolation and identification of the poliomyelitis group of viruses by means of tissue culture has stimulated interest in the application of the roller tube technic to viral and rickettsial agents in general(1-6). The above method involves imbedding fresh tissue in a chicken plasma clot and the cytologic examination of the fibroblast outgrowth at intervals. The use of a clot as a matrix for growing cells has a number of recognized disadvantages among which are listed the following: 1) preparation and preservation of plasma and chick embryo extracts is time-consuming and costly; 2) there is often clouding and liquefaction of the clot; 3) subcultures are tedious and exacting to prepare; 4) staining procedures often necessitate sectioning; 5) the composition of plasma and embryo extract is variable and chemically undefined; 6) plasma may contain contaminating viruses. Some of these objections may be circumvented by means of the glass wool roller tube technic herein described.

Methods. A very thin mat of glass wool† is cut into an approximately 2¼ by ¾ inch piece. This is placed between two metal blocks measuring 1¾ inches long and ¼ inch wide, and the free edges of the fibers are fused with a flame. The glass mat is then washed with ethyl ether and sterilized in the oven at 160°C for 1 hour. Heart or chorioallantoic membrane from a 10-day chick embryo is minced in a petri dish containing 1 part Simms' ox serum ultrafiltrate and 3 parts Hanks' balanced salt solution(5), and a concentration of 100 units of penicillin and 0.1 μg of streptomycin per ml. Three to 6 fragments of thoroughly washed tissue are arranged lengthwise along the inner surface of a pyrex glass tube measuring 16 x 150 mm. The glass wool mat is then placed over the tissue, pressed down gently and the tube cooled for a few seconds in an ice water bath. With a capillary pipette 2-3 drops of sterile, liquid, 2.5% washed agar-agar in saline are placed over the glass mat at the extreme top and bottom. The tube is again immersed in the ice bath in order to solidify the agar. This procedure serves to fix the mat to the sides of the tube and to trap the tissue beneath the glass wool. One

* Aided by a grant from the National Foundation for Infantile Paralysis.

† Pyrex Brand Filtering Fibre; Owen-Corning Fiberglas, Corning, N. Y.,

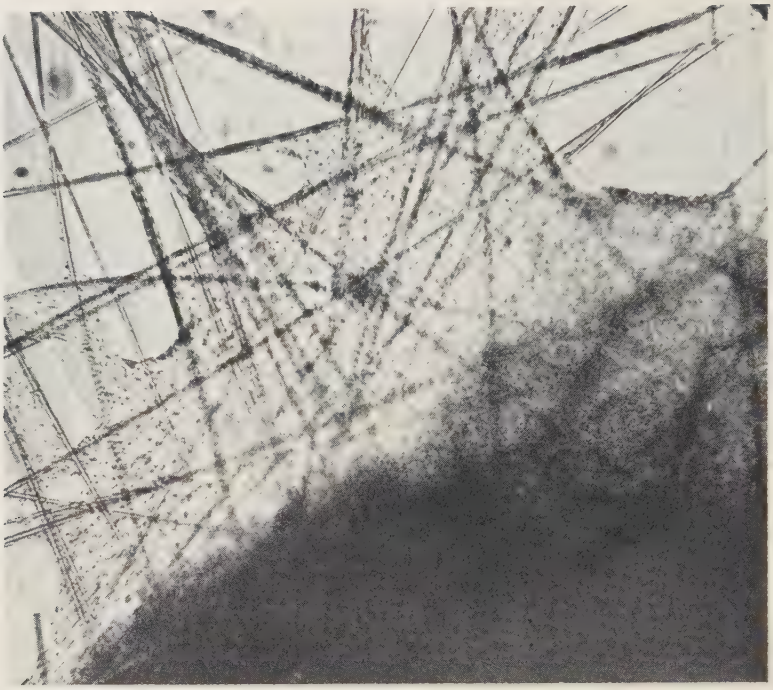

FIG. 1. Fibroblast outgrowth from a fragment of chick embryo heart under a glass wool matrix after 48 hours incubation at 35°C. Note the proliferation along the margins of the fibers and subsequent filling of interstices. × 65.

ml of nutrient fluid consisting of chick-embryo extract (1 part), unheated horse serum (1 part), Simms-Hanks 1:3 mixture (8 parts), and antibiotics in the concentration previously indicated is then added. The tubes are stoppered and incubated at the selected temperature in the roller drum. If a combination clot and glass wool matrix is desired the following procedure is employed. One drop of chicken plasma is added first and the tissue fragments are arranged in the plasma. The glass wool mat is pressed down over the tissue and 3 drops of chick embryo extract is dropped on the mat and spread over the surface. When clotting is complete the ends of the mat are fixed to the tube with agar-agar. Nutrient medium is then added and the tubes are stoppered and incubated.

Results. Experience with glass wool preparations has been largely confined to whole chick embryo, chorioallantoic membrane, chick-heart muscle, and human testicular tissue. The experiments have been designed to test the efficacy of the glass wool matrix as compared with the standard clot when an accepted type of nutrient medium is used. Under such conditions the fibroblasts begin to proliferate within 12 hours and grow out along the glass fibers. By 24 hours, a few of the spaces between fibers are filled with a film of cells, and by 48 hours, an irregular zone of growth approximately 1-2 mm in diameter may be observed (Fig. 1). If the nutrient fluid is changed as soon as the pH becomes slightly acid, the outgrowth gradually enlarges until the major portion of the matrix is covered with a thick sheet of fibroblasts.

Transplantation is accomplished by removing the glass wool mat to a petri dish containing balanced salt solution. The zone of outgrowth is cut with scissors into 2 mm squares which are then placed in a second tube. The explant is fixed to the side of the tube with a new glass wool sheet as previously described. Fresh nutrient fluid is added and the tube is returned to the rotator apparatus. If transplantation is successful, the fibroblasts invade the glass wool beyond the original explant within 48-72 hours.

Tissue outgrowths on glass wool mats lend themselves particularly well to staining procedures since portions can be removed, placed on slides and permitted to air dry. Any type of fixation and staining may be utilized depending upon the needs of the investigator. Permanent preparations are sealed under a cover slip and can be examined at leisure under all magnifications of the light microscope. In the thicker portions of the slide the cells are arranged in several layers and tend to be crowded together. At the periphery of the outgrowth the organization of the tissue and the cytology of the individual cell is clearly visible.

Discussion. The present communication is concerned with the substitution of the glass wool matrix for the plasma clot in the roller tube cultivation of chicken, human, and other tissues for reasons previously outlined. From the results obtained it seems likely that tissues which can be grown readily, such as chick embryo, will propagate as well on a glass wool matrix as they do along the surface of a glass tube. For more fastidious types of cells it may be necessary to incorporate the mat into a homologous or heterologous clot. Thus with human fibroblasts from adult testicular tissue, growth on glass wool has not been as luxuriant as with the chicken clot alone. This problem is being investigated.

Strips of perforated cellophane sheets which have been fixed to the side of the tube with agar-agar will also support outgrowth of fibroblasts from chick-embryo fragments. The cells, however, tend to disintegrate and cannot be visualized clearly because of artifacts in the cellophane. Furthermore, the cellophane mats are difficult to handle, slip along the sides of the tube, and fold over. Transplantation, therefore, is cumbersome. A comparison between cellophane and glass helices has already been reported by Shannon, Earle and co-workers(7,8). In these studies the glass proved as satisfactory for growing large numbers of cells as the cellophane. They also employed glass wool as a three dimensional substrate for mass cell culture with some success but discarded this matrix in favor of glass helices(9). A combined cellulose sponge and clot substrate has been described by Leighton(10) for the propagation of mouse mammary adenocarcinoma and chick embryo tissue. The method, however, does not permit observation of individual cells and requires sectioning prior to staining. Other types of matrices such as agar-agar, cotton, and starch particles have not supported fibroblast outgrowth under the experimental conditions imposed(11).

Summary. A simple tissue culture technic is described in which a thin glass wool mat, either alone or in combination with a plasma clot, is employed. The use of glass wool as a matrix for supporting growing tissue fragments circumvents some of the objections to a plasma clot in roller tube cultures.

The technical assistance of Virginia Jentoft is gratefully acknowledged.

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An Evaluation of the Analgetic Activity of the Dromoran Isomers. (19936)

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The relation between optical asymmetry and analgetic action has been investigated utilizing the optical isomers of methadone and some of its analogues. These studies have been reviewed by Chen(1). Denton and Beecher(2) carried out studies on human subjects, and concluded that d-methadone was inactive analgetically and d-isomethadone possessed an anti-analgetic action.

With the availability of the optical isomers of Dromoran, it was of interest to determine the relative analgetic activity of this pair of stereoisomers and the effect of d-Dromoran on morphine analgesia. The analgetic activity and the pharmacology of Dromoran have been described by Randall and Lehman(3), Gross, *et al.*(4), and Fromherz(5). The lack of addictive properties of d-Dromoran has been established by Isbell, *et al.*(6).

Methods. The effects of drug treatments were determined on five trained normal human subjects, using the Hardy-Wolff-Goodell technic. Pain thresholds were determined at 30 minute intervals until a maximal effect had occurred. The minimal intensity increment that the subjects were required to distinguish was equivalent to a 2.2% rise in pain threshold. The drugs dissolved in physiologic saline

TABLE I. Summary of Data.

Treatment	l-Dromoran tartrate	d-Dromoran "	d-Dromoran " and l-Dromoran "	dl-Dromoran hydrobromide	d-Dromoran tartrate	d-Dromoran " 30 min. before l-Dromoran tartrate	d-Dromoran " 30 min. before l-Dromoran tartrate	Saline placebo	Morphine sulfate	d-Dromoran tartrate and morphine sulfate	d-Dromoran tartrate 30 min. before morphine sulfate
mg	.5	.5	.5 .5	.8	1.0	.5 .5	1.0 .5		5	1 5	1 5
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
n	12	11	10	11	4	10	5	20	10	12	5
$\bar{x}$	10.9	.8	7.7	6.2	5.5	1.5	2.6	.09	8.1	4.2	3.1
$V_{\bar{x}}$	.61	.11	9.33	.25	.40	.54	.19	.06	1.08	.28	.29
$s_{\bar{x}}$	.78	.33	.57	.5	.63	.74	.04	.25	1.04	.53	.54

TABLE II. Statistical Analysis.*

Between treatments	1 vs. 3	2 vs. 5	2 vs. 8	3 vs. 4	3 vs. 6	4 vs. 6	9 vs. 10	10 vs. 11	5 vs. 8	5 vs. 7	5 vs. 10	5 vs. 11
d	3.2	.7	.1	1.5	2.2	.7	3.9	1.1	.6	1.1	2.7	1.6
V_d	.72	.65	.17	.58	.73	.65	1.36	.57	.60	.73	.82	.83
s_d	.85	.81	.41	.76	.85	.81	1.17	.75	.77	.85	.91	.91
"t"	3.76	.86	.24	1.97	2.59	.86	3.33	1.47	.78	1.29	2.97	1.76
N	20	19	29	19	12	13	20	15	28	13	20	13
P	.01–.001	.50–.30	.90–.80	.10–.05	.05–.02	.50–.30	.01–.001	.20–.10	.50–.30	.30–.20	.02–.01	.20–.10

* d = Difference between means. V_d = Variance. s_d = Stand. dev. N = No. of degrees of freedom. P = Probability.

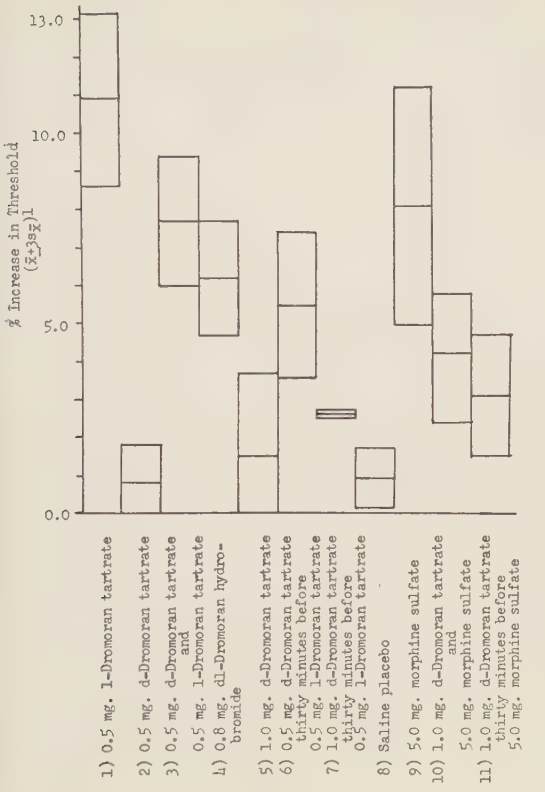

Fig. 1

Comparison of effects of drug treatment on the pain threshold.

[1]The line between each pair of blocks represents the mean (m) and the distance to the other end of each block is equal to three standard deviations ($3s_{\bar{x}}$).

were administered by subcutaneous injection. Drug treatments were randomized and neither the subject nor operator knew what drug combination each had received.

Results and discussion. The results are summarized in Table I, and a comparison of the effects of the drug combination is made in Fig. 1. Where visual inspection was insufficient to determine the significance of the data, the Null Hypothesis and Student's "t" Test were applied. These comparisons are summarized in Table II.

The drug treatments* tested were (1) 0.5 mg l-Dromoran† tartrate, (2) 0.5 mg d-Dromoran tartrate,† (3) 0.5 mg d-Dromoran tartrate and 0.5 mg l-Dromoran tartrate, (4) 0.8 mg dl-Dromoran hydrobromide,† (5) 1.0 mg d-Dromoran tartrate, (6) 0.5 mg d-Dromoran tartrate 30 minutes before 0.5 mg l-Dromoran tartrate, (7) 1.0 mg d-Dromoran tartrate 30 minutes before 0.5 mg l-Dromoran tartrate, (8) saline placebo, (9) 5.0 mg morphine sulfate, (10) 1.0 mg d-Dromoran tartrate and 5.0 mg morphine sulfate, (11) 1.0 mg d-Dromoran tartrate thirty minutes before 5.0 mg morphine sulfate.

The results of treatments 1, 2, and 5 show that the analgetic activity of Dromoran lies in the l-isomer. The d-isomer is able to diminish the analgetic activity of the l-isomer (compare treatments 1, 3, 4, 6, and 7). The results of treatments 9, 10, and 11 show that d-Dromoran is an effective inhibitor of morphine analgesia. A 30 minute interval between administration of the d-Dromoran and l-Dromoran or morphine does not appear to be highly significant (compare treatments 3 and 6 and 10 and 11)

The marked inhibition of the analgesia produced by l-Dromoran and morphine by d-Dromoran is evidence in support of the contention that these agents possess a similar mechanism of action. An extension of this type of "crossover" comparison of the effects of inactive or less potent optical isomers of analgetic compounds on the analgetic activity of its own stereoisomer and the active or more potent stereoisomer of members of other analgetic series would be an effective test of the following accepted although unproven contention: the mechanisms of action of the so-called "morphine-type" analgetics are similar.

Summary. 1. The relative analgetic potencies of the optical isomers and racemate of Dromoran in normal human subjects were determined. 2. The inhibitory effect of d-Dromoran on l-Dromoran analgesia and morphine analgesia was noted.

* The doses of dl-, d-, and l-Dromoran represent equimolecular doses on the basis of each of the stereoisomers.

† The supplies of Dromoran and its stereoisomers were supplied by Hoffmann-LaRoche, Nutley, N. J.

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Size of the Spores of *Histoplasma capsulatum.* (19937)

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Cozad and Furcolow(1) recently reported the results of their measurements of a large number of spores from several strains of *Histoplasma capsulatum*. These authors reported that most of the spores were small and nontuberculated. Since these findings were contrary to the usual concepts, it appeared worthwhile to make an independent check of their observations.

Materials and methods. Cultures of 5 of the isolates of *Histoplasma* (Traub, Spitzli, Guest, Perdue, and White) studied by Cozad and Furcolow were obtained, and spore counts of each strain were made. The isolates were grown in screw-top test tubes on potato dextrose agar slants. They were incubated at room temperature for approximately 6 months. In each instance the entire culture was scraped from the slant and was macerated by grinding in a sterile tissue grinder. Microslide preparations were made from each of the suspensions using lactophenol without the dye. Semi-permanent mounts were made by ringing the coverslips with a preparation of paraffin and vaseline. Microscopical observations were made with the high power oil immersion lens. Three traverse sweeps across each slide preparation were followed with no attempt to select areas, but all of the spores were counted as they appeared in the fields of observation. Therefore, differences in technics of Cozad and Furcolow's studies as compared to ours were as follows: 1) The cultures were approximately 6 months old when studied in our laboratory; their measurements were made on 1 and 5 months old cultures. 2) Our measurements were made with a 95x high power oil immersion lens (approximate magnification 1200 diameters); theirs were made with a 44x high dry lens (approximate magnification 500 diameters). 3) In our studies the entire culture was macerated by grinding in a sterile glass tissue grinder and the mounts then prepared from the suspension; theirs were made from a selected area of the culture. We hoped to secure, in this manner, a more representative sampling of the entire colony than could be obtained by the method of Cozad and Furcolow. We measured 1000 spores from each strain; they measured 250. Aside from the specific differences numerated above, technics and methods of observations were the same.

Results and interpretations. Fig. 1 presents a comparison of the results obtained in our laboratory to those obtained for the same strains of *Histoplasma* by Cozad and Furcolow(1). Although the curves shown in Fig. 1 are similar, our measurements show a greater frequency of small spores and fewer numbers of tuberculated spores. Only 6.8% of the 5000 spores measured by us exceeded 4.8 μ in diameter, and only 3.4% were tuberculated. This compares with 31.1% larger than 4.8 μ by their measurement and 11.2% tuberculated. It is also interesting to note that the largest percentage of tuberculated spores observed by us was 8.7% in strain 5, whereas strains 14 and 1 showed more than 20% tuberculated spores according to their measurements.

The greatest difference in measurements of the same strain occurred with strain 14 where

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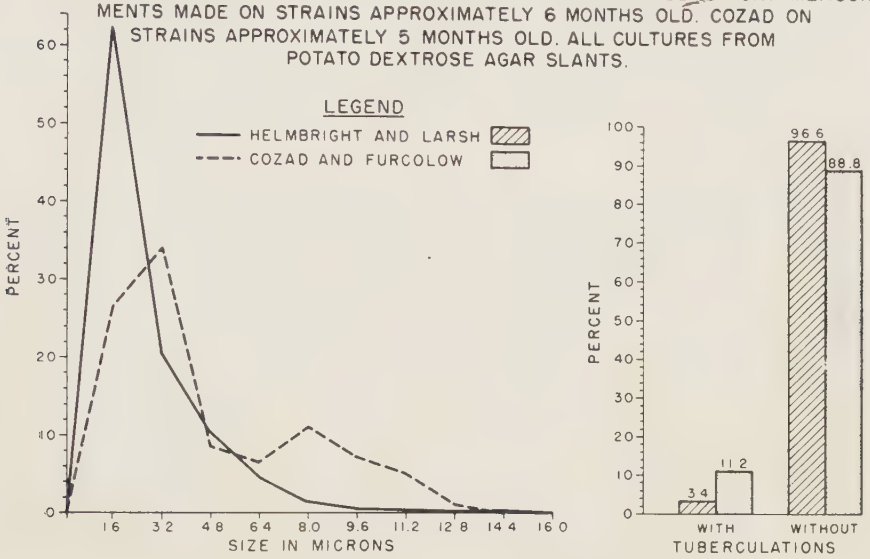
FIGURE 1

COMPARISON OF THE SIZE AND PRESENCE OF TUBERCULATIONS OF THE SPORES OF THE SAME 5 STRAINS OF *H. capsulatum* BY TWO OBSERVERS. HELMBRIGHT MEASUREMENTS MADE ON STRAINS APPROXIMATELY 6 MONTHS OLD. COZAD ON STRAINS APPROXIMATELY 5 MONTHS OLD. ALL CULTURES FROM POTATO DEXTROSE AGAR SLANTS.



we found 96.4% of the spores 4.8 μ or less in diameter, whereas the other workers found only 37.2% in this size range.

The nearest agreement of our measurements with theirs was noted in strain 2 where we found 95.2% of the spores to measure 4.8 μ or less, and they found 93.6% in this size range.

It appears that most of the discrepancies noted are due to the increased frequency of small spores recorded in our measurements. This we interpret to be due largely to the employment of the oil immersion lens in our measurements. In employing the oil immersion lens it was feasible to get greater accuracy in measurements, particularly of the small spores, which may account for the reported difference between our results and those of the other investigators. It is also interesting to note that we found no secondary rise in frequency of the spores about the 8 μ point compared to that reported by Cozad and Furcolow. Actually only 110 of 5000 spores measured (2.2%) were 8 μ or larger in size.

Summary. 1. In an effort to determine the size of the spores in *Histoplasma capsulatum*, 1000 spores from each of 5 strains were measured. To obtain a homogeneous suspension the entire culture was scraped from the potato dextrose agar slant and macerated in a sterile glass tissue grinder. The measurements were made using a high power oil of immersion lens. 2. On the whole, our results confirm those reported by Cozad and Furcolow and substantiate the high frequency of small spores in the strains of *Histoplasma capsulatum* observed.

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Effect of Terramycin on Fecal Microflora of Rats. I. Interrelation of Diet and Terramycin.* (19938)

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(Introduced by Edgar Zwilling.)

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The relationship of intestinal microorganisms to the well-being of the host has been extensively studied. Earlier experiments were designed to show the effect of various food substances on altering the intestinal flora from proteolytic saprophytes considered harmful to the host, to a supposedly beneficial saccharolytic flora(1-8). Supporting the original work of Cooper(9), much has been found to indicate that the intestines of man and of animals harbor bacteria capable of synthesizing nutrilites essential for the host. The growth-promoting effects of dietary sulfonamides and antibiotics have been extensively studied from this point of view(10-19). Few studies however, concerned the interrelation of dietary antibiotics and composition of diet upon intestinal flora of animals(20-23).

Considerable disparity exists in reports of changes in microflora, even using a single dietary antibiotic in comparable rations. The work here reported is a portion of the studies conducted to establish statistical significance of changes in microflora due to terramycin and in the influence of ration upon these changes. This was considered necessary in order to validate findings of changes in microflora as detected by available methods involving inherent fluctuations.

Methods and materials. Experimental animals. Thirty-six mature albino female rats were used.† Each animal was housed in a Hendrix cage, and received 30 g of appropriate ration every other day. In order to prevent spilling, deep 60 g opal ointment jars firmly fastened to each cage were used as feeding cups. Water was supplied *ad libitum*. The compositions of the rations fed are listed in Table I. Eighteen rats were placed on experiment April 1952. Of these, 6 received a "basal" ration, 6 received a "high protein low carbohydrate" ration and 6 received a "high carbohydrate low protein" ration. Of the 6 receiving each type of ration, 3 rats received in addition terramycin at the rate of 100 mg per kg of ration.‡ The other 18 rats were placed on experiment June 1952 and the above scheme was repeated.

Bacteriological examination. The feces of each animal were examined bacteriologically, first 2 days after initiation of treatment, and weekly thereafter for 5 weeks. Freshly excreted feces were taken from the 3 rats in each experimental group. A one gram sample of the composite feces was ground with sand in a sterile mortar and pestle, emulsified with 9 ml sterile 0.85% NaCl and the emulsion added to 90 ml of sterile water. From this initial suspension, serial dilutions were made. (Regular examination of this suspension for existence of protozoan infestation was discontinued after establishing that none of the animals examined was infected.) An estimate of the total viable anaerobes was made using B.B.L. eugon agar(23-25) incubated anaerobically at 37°C for 48 to 56 hours(27). A second set of similar pour plates was prepared using the same medium but incubated at 37°C for 48 to 56 hours to obtain aerobic count.

Lactobacillus acidophilus was enumerated in Difco tomato juice agar(28), incubated at 37°C for 48 hours in an atmosphere of approximately 10% CO_2. Total *Lactobacillus* counts as detected on the selective medium of Rogosa(29) are not reported pending identification of the isolations obtained in this laboratory. *Escherichia coli* population was enumerated on Tergitol-7 agar(30) to which was added 0.004% triphenyl tetrazolium chloride (T.T.C.)(31). Inoculation was made by spreading 0.1 ml amounts of the ap-

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† Rats were obtained through courtesy of Dr. R. B. Hubbell, Conn. Agri. Exp. Station, New Haven, Conn.

‡ Crystalline terramycin hydrochloride donated by the Chas. Pfizer and Co., Brooklyn, N. Y.

TABLE I. Composition of Rations.

	Basal ration (lb)	High protein, low carbohydrate, (lb)	High carbohydrate, low protein (lb)
Basic ingredients*	5.125	5.125	5.125
Casein	14.705	26.470	4.875
Dextrin	30.170	18.405	40.000
Total	50	50	50
Protein, %	24	45.9	9.10
Carbohydrate, %	73.8	51.9	88.7

* Basic ingredients for 200 lb of finished ration:

Alfalfa meal 17%	5 lb	$MnSO_4$	11.2 g
Dried butyl solubles (by 500)	4	Niacin	2
NaH_2PO_4	5.5	Vit. A dry 5000 I.U./g	80
Limestone	3.2	" D " 2000 "	20
Choline chloride (25%)	.5	Thiamin	40
B_{12} (Proferm) 12 mg	2	Pyridoxine	50 mg

TABLE II. Mean Values of Logs of All Enumerations of Total Anaerobes, Aerobes, *L. acidophilus*, *E. coli*, *Proteus spp.* and Fecal Streptococci, in Feces of Albino Rats on 3 Diets With and Without Terramycin. Statistical data include stand. error, ration difference, duplication of experiment and significant probabilities.

	Organisms					
Exp. groupings	Total anaerobes count $\times 10^6$	Total aerobes count $\times 10^6$	*L. acidophilus* count $\times 10^5$	*Proteus spp.* count $\times 10^2$	*E. coli* count $\times 10^3$	Fecal streptococci count $\times 10^3$
Rations						
Basal	2.850±.101	2.628±.092	2.748±.223	3.833±.247	3.872±.296	3.132±.237
Protein	2.741±.152	2.303±.177	3.022±.216	3.576±.255	3.500±.189‖	2.466±.317
Carbohydrate	2.770±.134	2.518±.113	2.594±.223§	3.058±.349	2.976±.199*	2.929±.286
Terramycin						
(—)	3.192±.072†	2.870±.109†	3.548±.094†	2.596±.163‡	3.382±.135	1.901±.164
(+)	2.382±.090	2.096±.108	2.028±.155	4.381±.206	3.517±.246	3.784±.215
Replication						
I	2.919±.110*	2.508±.104	3.018±.157	3.335±.245	2.976±.212†	3.328±.249*
II	3.982±.099	2.548±.116	2.558±.195	3.642±.231	3.924±.147	2.357±.219

* $P < .05$. † $P < .01$. ‡ $P < .001$. § 2 missing values calculated (60–2 = 58).
‖ 1 missing value calculated (60–1 = 59).

propriate dilution with a bent glass rod. The plates were incubated aerobically for 12 to 18 hours at 37°C. The differentiation of *E. coli* from other enterobacteria, including *Proteus spp.* is based on the inability of *E. coli* to reduce T.T.C. to a deep red formazan compound, whereas other coliforms regularly do so. Colonies of *E. coli* appear on this medium as large yellow colonies with deep orange centers. *Proteus spp.* were enumerated on urea ricinoleate medium(32) and incubated aerobically at 37°C for 24 hours.

All poured and smeared plates of appropriate dilutions were prepared in duplicate and the colonies arising counted with a Quebec colony counter and hand tally. Fecal streptococci were detected using Difco sodium azide medium(33). Five tubes of medium were inoculated with each appropriate dilution and incubated at 45.5°C for 48 hours in a double walled incubator equipped with a fan. When no growth was apparent in any of the tubes after 2 days incubation, the tubes were re-incubated for an additional 24 hours. From the numbers of positive tubes, the most probable number of each sample was determined from the tables of Prescott(34).

Statistical analysis. The data were subjected to standard statistical methods(35). Table II includes the mean values of the logarithms of the real numbers ± their standard errors and degree of variability of the

TABLE III. Mean Logs of 6 Weekly Values Including L.S.D. Among Subgroups of All Animals on Different Rations With and Without Terramycin.

Organisms	Experiment	Basal		High protein, low carbohydrate		High carbohydrate, low protein		L.S.D. for† sub groups
		Terramycin —	Terramycin +	Terramycin —	Terramycin +	Terramycin —	Terramycin +	
Total anaerobes	I	3.288	2.560	3.330	2.382	3.379	2.578	
$\times 10^6$	II	2.856	2.694	3.246	2.007	3.050	2.074	
	Mean	3.072	2.627	3.288	2.194	3.214	2.326	.440
Total aerobes	I	2.808	2.179	2.915	1.936	2.790	2.424	
$\times 10^6$	II	2.829	2.694	2.978	1.385	2.900	1.959	
	Mean	2.819	2.436	2.946	1.660	2.845	2.191	.720
L. acidophilus	I	3.586	2.262	3.875	2.823	3.163	2.397*	
$\times 10^5$	II	3.382	1.762	3.726	1.664	3.556	1.260*	
	Mean	3.484	2.012	3.800	2.244	3.360	1.829	1.102
E. coli	I	3.186	3.695	2.923	3.360	2.345	1.844	
$\times 10^3$	II	3.490	5.118	3.914	3.806*	3.938	3.277	
	Mean	3.338	4.406	3.413	3.583	3.391	2.560	.872
Proteus spp.	I	3.053	4.381	2.269	4.295	2.141	3.872	
$\times 10^2$	II	2.986	4.911	3.489	4.255	1.645	4.572	
	Mean	3.020	4.646	2.877	4.275	1.893	4.222	1.131
Fecal strepto-	I	2.815	4.373	2.072	4.351	1.726	4.630	
cocci $\times 10^3$	II	2.073	3.264	.748	2.693	1.970	3.392	
	Mean	1.222	3.819	1.410	3.522	1.848	4.011	1.210

* One missing value calculated (Snedecor 1946).
† L.S.D. computed at the 5% level of probability.

following experimental groupings: (a) among rations, (b) between composited treated and untreated animals, and (c) between replicates. Only the significant probabilities are included in Table II. Table III shows the two values making up each subgroup (mean logs of all enumerations) and the subgroup means. The least significant difference (L.S.D.) at the 5% level probability between subgroup means is also included in Table III.

Results and discussion. The individual groups of microorganisms studied will be discussed in the order of their appearance in the Tables II and III.

Total anaerobic count. The type of ration had no noticeable effect on the total anaerobic count, although the addition of terramycin to any diet caused a significant decrease ($P < 0.01$). This effect was of the same order without regard to the type of ration as evidenced by the absence of a significant interaction between ration and terramycin.

Total aerobic count. The total aerobic count was also reduced in numbers ($P < 0.01$) by the addition of terramycin to any ration. The type of ration *per se* had no effect on the total numbers of aerobes, nor was there an apparent interaction between diet used and effect of terramycin.

L. acidophilus. Contrary to the reports of many investigators, the ability of dextrin to maintain a high aciduric intestinal flora was not demonstrated in this work(3,7). This finding is in agreement with Nath(36). A statistically significant decrease ($P < 0.01$) in *L. acidophilus* count resulted from the addition of terramycin to any of the 3 rations; these reductions were comparable regardless of rations.

E. coli. No change in *E. coli* count was observed from any ration, or from the mean effect of terramycin. A decrease in bacterial numbers ($P < 0.10$) was noted with the terramycin supplemented high carbohydrate low protein ration; an increase ($P < 0.05$) in the case of the terramycin supplemented basal ration, and no significant change in the case of the terramycin supplemented high protein low carbohydrate ration.

Proteus spp. The type of diet was found to affect the concentration of *Proteus spp.* A higher count was obtained with the basal diet than with the high carbohydrate low protein ration ($P < 0.05$), but the count with high

protein low carbohydrate was not statistically higher than the basal diet. The addition of terramycin to any diet resulted in a comparable increased *Proteus* count. ($P < 0.05$). Thus it is seen that an increase in *Proteus* numbers was encountered regardless of the numbers of *E. coli*, which is contrary to the findings of Seiburth(26).

Fecal streptococci. No interaction between diet and terramycin and no effect of diet *per se* were found to alter the count of fecal streptococci. The inclusion of terramycin, however, in any diet increased the numbers of fecal streptococci ($P < 0.001$).

Summary. 1. A study was made of changes in fecal microflora of albino rats engendered by changes in composition of diet, and by incorporation of terramycin in the 3 diets employed. The data were statistically analyzed as to ration effect, drug effect, interaction between ration and diet and reproducibility of experiments. 2. Diets *per se*, showed little or no effect in modifying the counts of total anaerobes and aerobes, *L. acidophilus*, or fecal streptococci. Nor was there a statistically significant interaction between terramycin and diet in these groups of organisms. Terramycin caused significant reductions of anaerobes, aerobes, and *L. acidophilus* and a marked increase of fecal streptococci regardless of ration. 3. The use of tergitol-7-agar containing T.T.C. was described for enumeration of *E. coli* in feces. The counts of *E. coli* were not affected by diet or terramycin *per se*, but a high degree of interaction between diet and terramycin obtained. The action of terramycin on the *E. coli* count was dependent on the type of ration fed, especially as evidenced by significant differences between terramycin supplemented high carbohydrate low protein ration and terramycin supplemented basal ration. 4. The high carbohydrate low protein diet resulted in a much lower count of *Proteus spp.* than either of the other 2 rations. Supplementation of all rations with terramycin resulted in a marked increase in numbers of *Proteus spp.* It was especially noted that the effect of terramycin was independent of the ration fed in this case.

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Effect of Actidione on Growth and Respiration of *Myrothecium verrucaria.** (19939)

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Actidione produced by *Streptomyces griseus*(1) is one of the most active antibiotics toward yeasts and filamentous fungi(2,3). As with other fungicides, however, there is little or no evidence on the nature of its action. Since penicillin and streptomycin are known to interfere with respiratory metabolism(4), it was desirable to compare the effects of actidione on fungus growth and respiration. This was done by measuring the inhibition of germination and of spore and mycelial respiration of *Myrothecium verrucaria* under various conditions.

Methods. Strain QM460 of *Myrothecium verrucaria* from the Quartermaster General Laboratories was cultured at 30°C on Whatman No. 2 filter paper on a modified Fries medium of Sinden, Mix and Siu(5). Point inoculation was used for routine transfer to avoid sector variants and spore suspension inoculation was used for rapid production of spores of uniform age (Mandels and Norton)(6). Mycelial pellets, 0.2 to 0.5 mm in diameter were produced in a 24-hour growth period at 30°C on a rotary shaker, essentially by the technic of Darby and Goddard(7). Percentage germination was measured at 30°C on glass slides in drops of the Mandels and Norton(6) nutrient solution. Counts of 300 to 500 spores were taken after 4 to 5 hours from 4 fields in each of 3 drops. Controls regularly germinated 99 to 100% so that corrections for natural mortality were not required. Actidione† solutions were prepared in serial dilution with a dose ratio of 1.1 which provided 4 or 5 levels of inhibition between 1 and 99%. LD50 values were determined graphically from dosage response curves on log-probability coordinates. *Respiration measurements* were made in air in standard Warburg apparatus at 30°C using the "direct" method(8) for CO_2 with correction for bound CO_2. Two to 4 mg dry weight of washed spores or mycelium was used per flask. Spores were suspended in nutrient solution(6) containing

TABLE I. Effect of Actidione on Germination and on Respiration of Spores and Mycelium of *M. verrucaria.*

	No. of exp.	LD50 values in p.p.m. Mean ± S.E.
Germination	5	2.71 ± .20
Spore resp.* (O_2 consump.)	14	.74 ± .14
Mycelium resp.* (O_2 consump.)	4	8.03 ± .17

* Period of maximum sensitivity: ca. 30-60 min. for spores, 2nd or 3rd hr for mycelium.

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† Actidione (reagent grade) was kindly supplied by J. H. Ford, The Upjohn Co., Kalamazoo, Mich.

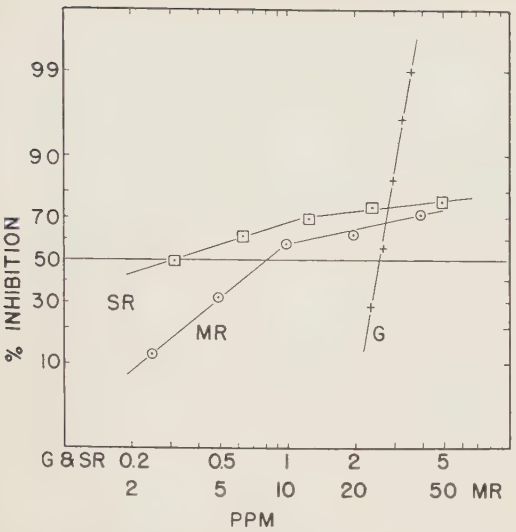

FIG. 1. Typical dosage-response curves on log-probability coordinates for spore O_2 consumption (SR), mycelial O_2 consumption (MR) and germination (G).

mineral salts, glucose and yeast extract and the mycelial pellets in pH 6.2 phosphate containing Mg and glucose(7). Actidione concentrations were chosen to give 3 to 5 levels of inhibition and LD50 values were estimated as in the case of germination.

Results. Table I gives mean LD50 values and standard errors for germination and for spore and mycelial respiration of *M. verrucaria*. Dosage-response curves for germination (Fig. 1) were linear throughout the measurable range of inhibition and were very steep. Mean values for this range can be taken as from 2 p.p.m. (LD1) to 3.6 p.p.m. (LD99). On the basis of LD50 values spore respiration was almost 4 times as sensitive to actidione as germination during the period of maximum sensitivity. This period was usually from 30 to 60 min. after addition of spores to nutrient and toxicant. Individual LD values for spore respiration varied considerably more than those for germination or mycelial respiration. This was due partly to the effect of the low slopes of the curves (Fig. 1) on the accuracy of estimating LD50 and partly to the rapid change in inhibition of spore respiration with time which is discussed below. It is also apparent that spore respiration was about 10 times as sensitive to actidione as mycelial respiration.

Representative data in Table II show that inhibition of spore respiration decreased rapidly after the first hour and disappeared completely after 3 or 4 hours at actidione levels causing initial inhibitions up to 50 to 60%. Three possible reasons were investigated: 1) decrease in spore sensitivity during the early stages of growth; 2) spontaneous decomposition of actidione; and 3) detoxication of actidione by the spores. The first possibility was tested by comparing the inhibition by 0.7 p.p.m. of actidione added at the start and after 200 min. incubation of the spores in the Warburg. The respective % inhibitions were 35 and 0 indicating a marked decrease in sensitivity during the first 3 hours. Spontaneous decomposition was tested by incubating actidione in nutrient without spores for 4 hours before measuring respiratory inhibition. Results of a typical experiment in Table III show that there was no decomposition. Detoxication was tested as follows: actidione was incubated with spores for a 4-hour period, the spores were removed by centrifugation, a fresh batch of spores was added with additional nutrient, and the respiratory inhibition measured. The results in Table III show that there was extensive detoxication or removal of actidione from the medium during the first 4-hour period in which spore respiration had recovered completely at the two lowest concentrations and about 80% at the highest.

At concentrations of actidione above 1 p.p.m., which gave initial inhibitions of spore respiration greater than 50 to 60%, the inhibition increased with time reaching a maximum of 80 to 85% (Table II and Fig. 1) which remained constant from about 2 to 20 p.p.m. The % inhibition of respiration at 2 p.p.m. where inhibition of germination just began (LD1) ranged from 65 to 80 with an average of about 75% for 9 trials.

The respiratory quotient for untreated spores averaged 0.96 and 1.03 for the first and second hours, respectively. Comparison of inhibition of O_2 consumption and CO_2 evolution of spores is shown in Table IV. No significant differences in % inhibition were found over a range of concentrations and times of exposure.

TABLE II. Change in % Inhibition of O_2 Consumption of Spores and Mycelium of *M. verrucaria* with Time.

Exp.	Conc. of actidione in p.p.m.	Hr after treatment 1st	2nd	3rd	4th
Spores 2-2A	.20	45	17	5	—3
	.40	58	31	25	2
	.60	62	52	36	22
1-11	.63	51	47		
	1.25	57	71		
11-15	1.25	74	81		
	2.50	78	84		
Mycelium 1-12	1.25	26	12	3	
	2.50	51	37	30	
	5.0	45	45	37	
	10.0	50	52	58	
1-24	10.0	42	48	51	49
	25	43	49	63	76
	50	48	51	65	83

TABLE III. Detoxication of Actidione by Spores of *M. verrucaria* Measured by % Inhibition of O_2 Consumption (First Hour).

Conditions	Conc. in p.p.m. .51 %	.34 %	.17 %
Fresh actidione standard	52	46	33
Actidione incub. without spores*	54	48	34
Actidione incub. with spores*	24	6	—6

* Actidione incubated under same conditions, with or without spores, for 4 hr before inhibition measurement.

TABLE IV. Relative Effect of Actidione on O_2 Consumption and CO_2 Evolution of Spores of *M. verrucaria* (First Hour).

Exp.	Conc. in p.p.m.	Hr after treatment	Inhibition O_2 %	Inhibition CO_2 %
1-25	1.4	1	75	76
		2	81	82
		3	78	81
1-26	.7	1	67	53
		2	61	63
		3	33	37
	1.4	1	73	69
		2	80	80
		3	73	77
2-14	.5	1	61	61
	1.0	1	70	66
	1.5	1	73	75

Mycelial respiration showed changes in % inhibition with time similar to those of spores, but at higher concentrations (Table II and Fig. 1). Again a maximum inhibition of about 85% was reached, in this case between 50 and 100 p.p.m. of actidione.

Discussion. Although no dosage-response curves or LD50 values for actidione effects on germination of other fungi have been reported, *M. verrucaria* appears to be roughly intermediate in sensitivity as compared with the species tested by Whiffen(2), Wollen, Sutton and Skolko(3) and others. On the other hand, actidione is one of the more active fungicides tested on *M. verrucaria,* although lower LD50 values have been found for ethyl mercury chloride(9) and 2,3-dichloro-1,4-naphthoquinone(10).

In interpreting the action of actidione on *M. verrucaria* it is significant, first, that spore respiration is much more sensitive than mycelial respiration and that both show recovery with time. In the case of spores the recovery is complete in 3 or 4 hours if the initial inhibition is not over 50 to 60%. During this period the sensitivity of spore respiration was shown to decrease, apparently approaching that of mycelium as germination progressed. At the same time spores removed actidione from the medium. Whether this removal was due to actual detoxication and thereby contributed to the decrease in respiratory inhibition, or whether the removal merely paralleled the decrease in respiratory sensitivity, can not yet be decided. Klomparens(11), however, has reported that mycelium of *Poria microspora* will take up actidione from solution and that it cannot be detected in the mycelium by bioassay after drying. It is possible, therefore, that both decreasing sensitivity and detoxication were involved in the recovery.

The most unusual property of actidione, however, is its greater toxicity to spore respiration than to germination. It was the only compound showing this property among 6 organic fungicides tested on *M. verrucaria* (9) including ethyl mercury chloride, 2,4-dinitrophenol, disodium ethylenebisdithiocarbamate, tetramethylthiuram disulfide, and pentachlorophenol. Klöpping(12) also found only one fungicide out of 10 which may have been more toxic to respiration than to growth of *Aspergillus niger* and *Penicillium italicum.* The precision of his methods, however, makes

even this case doubtful.

In the present work about 75% of spore respiration was inhibited by actidione levels (2 p.p.m.) which did not interfere with germination. At higher levels (above 3.6 p.p.m.), where germination was completely inhibited, the maximum inhibition of respiration was about 85%. This may indicate that only roughly 10% of respiration is required for growth. A similar conclusion was reached by Commoner and Thimann(13) for the effects of iodoacetate on *Avena coleoptile*. However, the present manometric data alone can not establish a direct connection between inhibition of growth and respiration. Until this is done other interpretations of the per cent of respiration linked with growth are possible.

Conclusions. The effect of actidione on the germination and the spore and mycelial respiration of *Myrothecium verrucaria* was measured by dosage-response curves and LD50 values and by change in per cent inhibition with time. Spore respiration was about 4 times as sensitive as germination and 10 times as sensitive as mycelial respiration. Only a small fraction of respiration seemed to be required for growth. Inhibition of spore and mycelial respiration decreased with time, in the spores because of decreasing sensitivity and possibly also because of detoxication.

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Effects of 2,4-Dinitrophenol Concentrations on Rates of Respiration and Fermentation of Yeast.* (19940)

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Most of the interest in the action of 2,4-dinitrophenol (DNP) on metabolism has stemmed from its ability to stimulate respiration(1,2), to prevent assimilation and other synthetic processes(3), and to interfere with the coupling of phosphorylation and respiration(4-6). It has also been noted that higher concentrations of DNP inhibit respiration of glucose(1) and acetate(7) and, under anaerobic conditions, the fermentation of glucose(8). However, the observations concerning inhibition of metabolism were incidental to studies pertaining to other actions of DNP. In the present paper quantitative observations are presented concerning marked differences in the inhibitory action of DNP on respiration as compared to fermentation, with glucose as substrate. Comparisons are also made with the stimulating actions of DNP on endogenous and exogenous respiration.

* This paper is based on work performed under contract with the U. S. Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

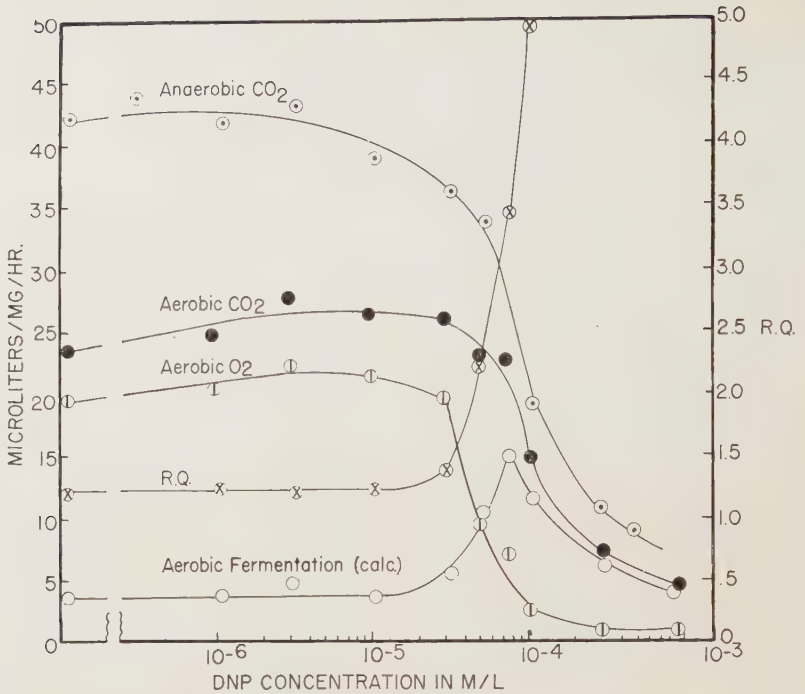

FIG. 1. Effects of various concentrations of DNP on rates of respiration, anaerobic and aerobic CO_2 production, and aerobic fermentation, with glucose as a substrate. Each Warburg flask contained 10 mg of yeast in 3 ml suspension at pH 3.5, at 30°C. Initial glucose conc. was .2 M.

Methods. Fresh Baker's yeast (Standard Brands, Inc.) was thoroughly washed. In washing, centrifugation was carried out for only 3 minutes at about 1000 X gravity, so that only the heavy viable cells were carried down. Colloidal materials and cell debris were thereby discarded. The cells were then starved for 2 to 3 hours with aeration in order to attain the steady-state condition in regard to endogenous metabolism(9). All measurements of CO_2 production and oxygen consumption were carried out by standard Warburg technic. The pH of the yeast suspension and of all solutions was adjusted to pH 3.5. At this pH, fermenting yeast is self-buffering(10) and the action of DNP is maximal(1).

Results. DNP was found to be a potent inhibitor of respiration. Concentrations as low as 2 to 3 x 10^{-5} M produced a definite inhibition; 5 x 10^{-5} M produced 50% inhibition; and 2 x 10^{-4} M produced virtually 100% inhibition (Fig. 1). On the other hand, if CO_2 production rather than O_2 consumption was measured, also under aerobic conditions and with glucose as a substrate, the inhibition curve was not the same as for respiration, but was shifted considerably toward higher DNP concentrations. Thus, no inhibition was observed until the DNP concentration was at least 5 x 10^{-5} M. An inhibition of 50% required 1 x 10^{-4} M. Even at 1 x 10^{-3} M DNP,† there was only 80% inhibition. In terms of the concentration of DNP necessary to produce 50% inhibition, O_2 consumption was twice as sensitive to the inhibitor as was aerobic CO_2 production.

Aerobic CO_2 production by yeast from glucose is the sum of the CO_2 associated with respiration and that associated with aerobic fermentation. If it is assumed that the R.Q. for respiration of glucose is 1.0, then respiratory CO_2 is equal to the O_2 consumption. The CO_2 associated with aerobic fermentation can then be calculated by subtracting the respiratory CO_2 from the total aerobic CO_2. Normal-

† Concentrations of DNP higher than 1 x 10^{-3} M could not be used because of the limited solubility of this compound at pH 3.5.

TABLE I. Effect of Glucose Concentration on Stimulating Action of DNP on Respiration. Temp., 30°C; pH, 3.5; yeast conc., 10 mg/flask; DNP conc., 1×10^{-5} M. All determinations were in duplicate.

Glucose conc., M/L	Rate of respiration			
	No DNP, μl/mg/hr	DNP, μl/mg/hr	Increase due to DNP, μl/mg/hr	Stimulation, %
.0	.40	2	1.6	500
.001	2.8	5.7	2.9	103
.003	11.2	15.8	4.6	41
.005	14.6	19.8	5.2	36
.01	16.3	20.5	4.2	25
.1	19.1	23.3	4.2	22

ly baker's yeast has a significant level of aerobic fermentation even with maximal oxygenation. For example, in the experiment of Fig. 1 the R.Q. was 1.2. The rate of aerobic fermentation was 3.5 μl/mg/hr, or somewhat less than 10% of the rate of anaerobic fermentation. In the presence of DNP, in concentrations above 3×10^{-5} M, there was a dramatic increase in the R.Q. The rate of aerobic fermentation increased five-fold, from a normal of 3.5 μl/mg/hr to a peak at 15 μl/mg/hr at 8×10^{-5} M DNP.

The inhibition curve for anaerobic fermentation was somewhat parallel to that for aerobic CO_2 production, with 50% inhibition in each case requiring a DNP concentration of 1×10^{-4} M, twice as high as that required for 50% inhibition of respiration. The appearance of an elevated rate of aerobic fermentation seemed to be related to this relative insensitivity to DNP of fermentation as compared to respiration. For example, those concentrations of DNP (3 to 8×10^{-5} M) which gave an appreciable inhibition of respiration but which had little effect on fermentation induced a high rate of aerobic fermentation. In fact, the increase in the rate of aerobic fermentation was roughly proportional to the decrease in the rate of respiration. On the other hand, those concentrations of DNP (above 8×10^{-5}) which were associated with a marked inhibition of anaerobic fermentation, resulted in a parallel diminution in the rate of aerobic fermentation.

Concentrations of DNP lower than those required to inhibit respiration, resulted in a small but definite stimulation of respiration. In Fig. 1, this may not be too apparent, but in other experiments, a consistent stimulation of about 20% was observed. The stimulation of respiration on a percentage basis was much more evident when the concentrations of glucose were lower (Table I), with by far the greatest effect, a 500% increase, in the complete absence of glucose. However, on an absolute basis, the increased oxygen consumption was greater in the presence of higher concentrations of sugar. Thus the stimulation of respiration by DNP seems to be in part due to an increased endogenous rate and in part due to an increased respiration of glucose.

The effects of DNP on endogenous respiration are shown in more detail in Fig. 2. A definite stimulation was observed at concentrations of DNP as low as 3×10^{-6} M, but the maximal effect, a 700% increase, was found at 5×10^{-5} M DNP. Higher DNP concentrations were associated with decreasing rates of respiration, with complete disappearance of any stimulating action at 1×10^{-3} M. In terms of the concentrations of DNP required, the inhibition of the elevated endogenous respiration (Fig. 2) is markedly similar to the inhibition of the respiration of glucose (Fig. 1).

The stimulation of endogenous respiration by DNP was further characterized by studies with carbon monoxide (CO). Respiration of glucose by yeast is sensitive to CO, with reversal of the inhibition by light(11). On the other hand, endogenous respiration of starved yeast is normally insensitive to CO (12). Fig. 3 indicates that the increment of endogenous respiration induced by DNP is sensitive to CO, with reversal by light, re-

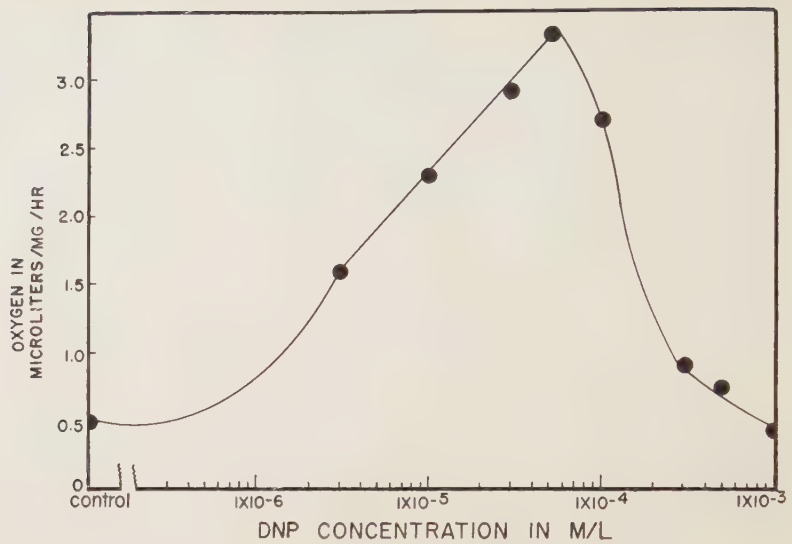

FIG. 2. Effect of DNP on endogenous aerobic metabolism. Each Warburg flask contained 40 mg of yeast in 3 ml suspension at pH 3.5, at 30°C.

sembling the respiration of glucose in this respect, rather than the normal endogenous respiration.

Discussion. The action of DNP on the rates of metabolism is complex, and obviously involves an interaction of DNP with several different enzyme sites at different concentrations of DNP. Low concentrations (1×10^{-6} to 1×10^{-5}) stimulate both endogenous and exogenous metabolism. Such concentrations are also associated with the inhibition of assimilation(3), phosphate uptake(13), potassium uptake(10) and many other synthetic activities. These effects are undoubtedly associated with the action of DNP in "uncoupling" phosphorylation from respiration(4-6). The experiments with carbon monoxide indicate that the endogenous respiration, which normally proceeds by a CO-insensitive system, is not only stimulated to a marked degree, but is shifted into CO-sensitive pathways, presumably a pathway involving the cytochrome system.

Higher concentrations of DNP (3×10^{-5} M to 1×10^{-4} M) inhibit respiration probably by acting on cytochrome reductase(14) but not the cytochrome system(15). Parallel to the inhibition of respiration, there was a diminished Pasteur effect and a marked increase in aerobic fermentation. DNP does not differentiate between respiration and the Pasteur effect, the inhibition in each case being about the same (the Pasteur effect was quantitated in terms of the Meyerhof Quotient)(16).

The highest concentrations of DNP (8×10^{-5} M to 1×10^{-3} M) inhibit both the aerobic and anaerobic fermentation of glucose, but at the same time induce a fermentation of endogenous stores(17). It is not known by what mechanism these effects are produced.

Summary. Low concentrations of DNP stimulate the respiration of living yeast cells in the presence or absence of glucose. Respiration in the absence of substrate is normally insensitive to CO, but the increment increase due to DNP is sensitive to CO. Higher concentrations of DNP inhibit both respiration and fermentation of glucose. However, the respiratory pathway is considerably more sensitive to the inhibitor than is the fermentation pathway. In consequence, at certain concentrations of DNP, the Pasteur effect is depressed and there is a striking increase in the rate of aerobic fermentation.

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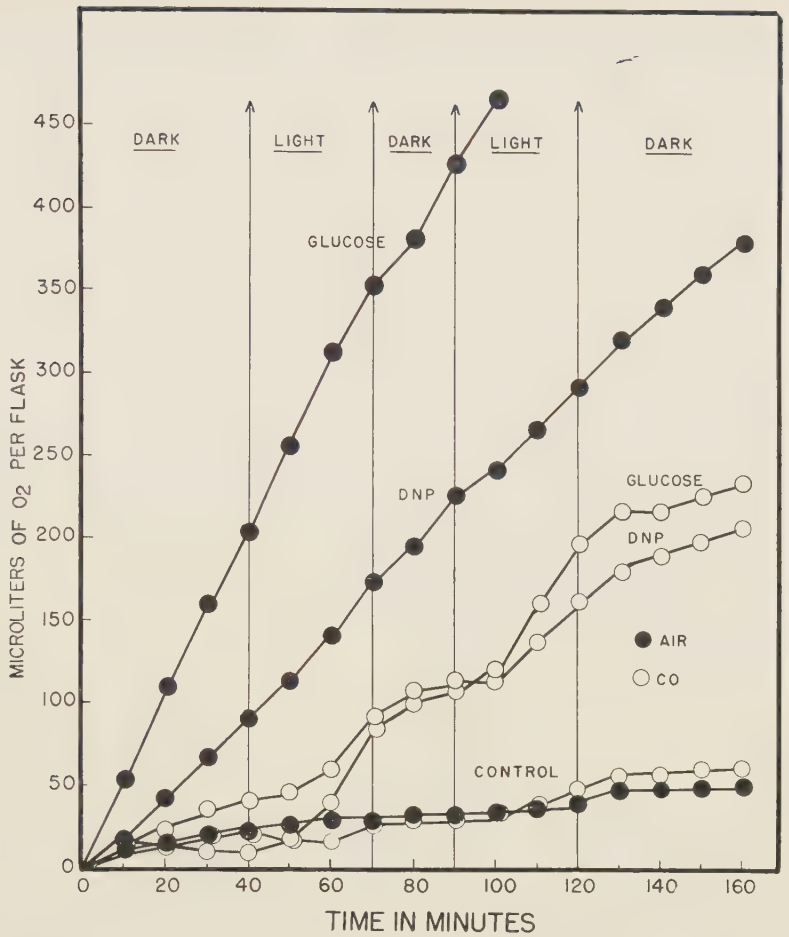

FIG. 3. Effect of CO on DNP-stimulated respiration in the dark and in the light. Each Warburg flask contained 40 mg of yeast in 3 ml of suspension at pH 3.5, at 30°C. Initial glucose conc. was .1 M and the DNP, 5×10^{-5} M. Gas mixtures were air or 95% CO – 5% O_2. Lights were 3 #2 photofloods approximately 50 cm from flasks.

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An Interrelationship between Calcium Ion and Serine in the Nutrition of *Lactobacillus casei.** (19941)

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The recent finding that D(-)-lactic acid is an important growth stimulant for *Lactobacillus casei*(1) prompted an investigation of the effect of calcium DL-lactate on the response of this organism to amino acids. A marked stimulation in response to serine was obtained with the addition of this compound to the test medium, but improvements in response to nine other essential (for *L. casei*) amino acids were only slight or negligible. Further investigation revealed that an elevated calcium ion concentration was essential for *L. casei* in media containing relatively low serine levels and that responses to serine and/or calcium were unaffected by additions of sodium DL-lactate.

Experimental. The basal test medium was the same as that used previously(1) except that the casein digests were replaced by 20 mg % each of DL-alanine, L-arginine monohydrochloride, L-aspartic acid, glycine, L-glutamic acid, L-histidine monohydrochloride monohydrate, DL-isoleucine, L-leucine, DL-lysine monohydrochloride, DL-methionine, L-proline, DL-phenylalanine, DL-serine, DL-threonine, L-tyrosine and DL-valine. The response of *L. casei*† to each of the amino acids found previously to be required by this organism(2) was determined in the basal medium (omitting in each case the test amino acid) alone and with additions of calcium chloride and sodium DL-lactate. The experimental procedures were the same as those described previously for amino acid determinations(3).

Results and discussion. Responses to arginine, leucine, phenylalanine, tyrosine and valine were not significantly affected by the presence or absence of calcium chloride and/or sodium DL-lactate at concentrations of 0.001 molar in the basal medium, and only slight stimulatory effects were noted in the responses to cystine, glutamic acid, isoleucine, and tryptophan when the supplements were used. The response to serine was unique, however, in that a marked stimulation was obtained by adding calcium chloride to the basal medium (Fig. 1). Sodium DL-lactate had no effect on response to serine in the basal medium alone and caused no further stimulation in the calcium chloride supplemented medium.

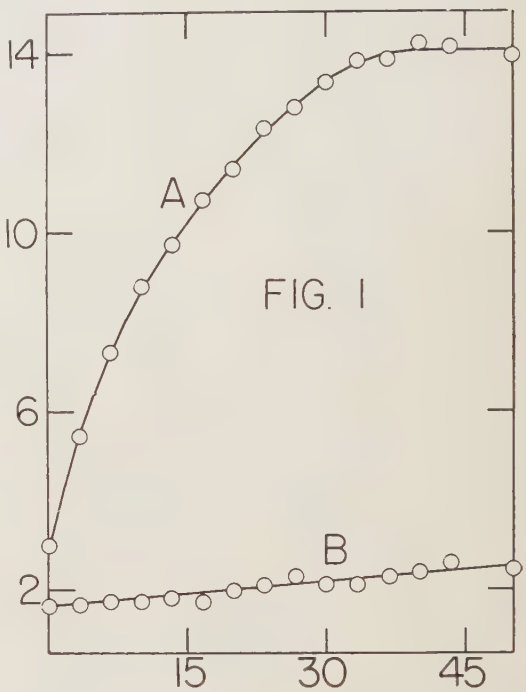

FIG. 1. Response of *L. casei* to DL-serine with 3 days incubation in A) the basal medium supplemented with .001 mM $CaCl_2$/ml, and B) the unsupplemented basal medium. The values on the vertical scale are the ml of .01 *N* NaOH required to titrate one ml of culture, and those on the horizontal scale are the concentrations of DL-serine in μg/ml.

Response to calcium was determined in the basal medium modified to contain a reduced concentration (5 mg %) of DL-serine.

* Paper 92. This work was aided by grants from Swift and Co., U. S. Public Health Service and the University of California.

† American Type Culture Collection No. 7469. This culture was maintained as described previously (3).

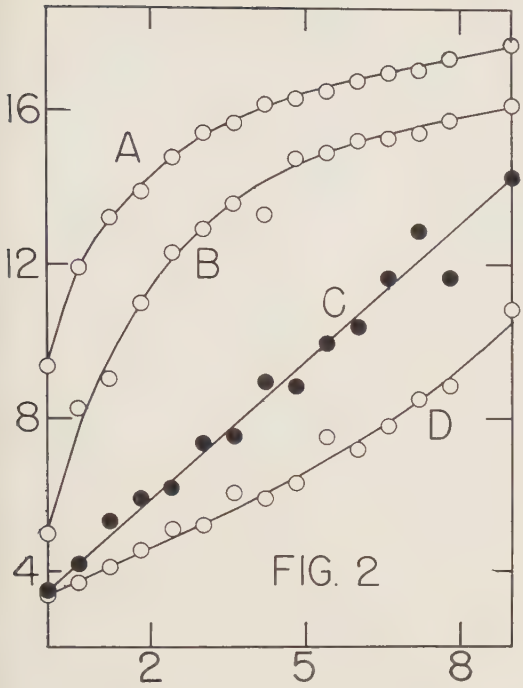

FIG. 2. Response of *L. casei* to $CaCl_2$ with 3 days incubation in the basal medium modified to contain 5 mg % of DL-serine and the following concentrations of sodium (in mM %): A) 7.1, B) 13, C) 25, and D) 36. The vertical scale is the same as in Fig. 1. The values on the horizontal scale are the concentrations of $CaCl_2$ calculated as μg of Ca/ml.

Under these conditions growth was poor in the absence of added calcium, but good growth was obtained with moderate additions of this ion,‡ (Curve C, Fig 2). In separate experiments it was shown that further slight stimulations of growth were obtained with still higher calcium concentrations up to those at which salts precipitated from the medium.

Mutual antagonisms between the ions of calcium, sodium and potassium are recognized in the biochemistry of higher organisms, and it seemed possible that the requirement of *L. casei* for calcium in the low-serine medium might be dependent upon the relatively high sodium content (24.6 mM %) of this medium.§ Accordingly the response to calcium was redetermined under the same conditions except that the sodium content was varied form 7.1 - 36 mM % in a series of 6 media.‖

A marked antagonism between sodium and calcium was evidenced by an increased slope of the calcium response curve at the lowest sodium concentrations (Curves A and B, Fig. 2) and by a decreased slope at higher sodium concentrations¶ (Curves C and D, Fig. 2). It appeared, however, that the calcium requirement was not dependent upon an excessively high sodium ion concentration.

No explanation of the unique relationship between serine and calcium is apparent at this time, and further investigations of the calcium metabolism of *L. casei* are in progress.

Summary. A requirement of *L. casei* for calcium was induced by employing a reduced serine concentration in the basal medium. The calcium requirement was not demonstrable with a reduced concentration of any one of the remaining essential (for *L. casei*) amino acids. A marked increase in the sensitivity of response to calcium was obtained with reduced sodium concentrations, but the requirement for calcium in the low-serine medium persisted even at the lowest test levels of sodium.

‡ The basal medium contained about 0.2 μg of calcium (as calcium DL-pantothenate) per ml.

§ This relatively high sodium chloride concentration is routinely used in media for studies in this laboratory which might lead to applications as assay methods. The use of a high basal salt concentration makes it possible to eliminate disturbing salt effects by compensating for the high salt concentrations which often are encountered in test samples(3).

‖ Sodium chloride and ammonium chloride were eliminated from the basal medium, and part of the sodium acetate was replaced by ammonium acetate (retaining the original acetate and ammonium concentrations) to obtain the initial sodium concentration of 7.1 mM %.

¶ An increased scattering of points may be noted in curves C and D of Fig 2. It seems likely that this effect may be due to variations in calcium dependence brought about by differing degrees of adaptation or mutation occurring in the separate assay tubes, since adapted cultures of *L. casei* which are capable of rapid and profuse growth in low-serine media without additions of calcium have been readily obtained under the conditions of curve C, Fig. 2 (unpublished data of D. E. Atkinson with the present authors). If this explanation is correct, it would appear that the adaptations were favored by the higher sodium concentrations.

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The Distribution of C^{14} Labeled Isonicotinic Acid Hydrazide in Normal Mice.* (19942)

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Isonicotinic acid hydrazide and its isopropyl derivative are now being subjected to widespread clinical evaluation throughout this country(10). The promise that these drugs have shown warrants a further study of their pharmacological properties, whether or not these drugs do become accepted generally for use in the treatment of tuberculosis. This paper is the first report of our group on a series of investigations to determine such properties of isonicotinic acid hydrazide. As a basis for interpreting future work, we have studied the absorption, distribution and excretion of the drug in normal mice.

The isotope tracer technic was used in this problem since it is a rapid yet sensitive technic well suited to determining micro quantities of a drug and its metabolite in many tissues. We have been able to obtain isotopically labeled isonicotinic acid hydrazide through the cooperation of the Health Division, Los Alamos Scientific Laboratory, Los Alamos, New Mexico. The drug which was synthesized by Murray and Langham(1) is labeled with C^{14} in the carboxyl position and has an activity of 0.045 mc/mg.†

Materials and methods. A saline solution of the labeled isonicotinic acid hydrazide diluted with crystalline normal drug was made up to contain 400 micrograms per milliliter with an activity of 2.54×10^6 cpm. The refrigerated solution was found by paper chromatography to be stable for longer than one month. Forty-two female mice of the CF_1 strain weighing approximately 20 g each were used for this study. Each mouse received a subcutaneous injection in the right hind leg of 0.5 ml of the above solution (10 mg/kg). The mice were kept in all-glass metabolism chambers(2) throughout the experimental period and the exhaled CO_2 trapped in towers of sodium hydroxide solution. At the end of each experimental period, the mice were sacrificed by cervical fracture, the heart rapidly exposed and 0.25 to 0.5 ml of blood withdrawn by cardiac puncture. The tissues were dissected out, weighed and aliquots plated directly in duplicate(3) on copper discs as 10% water homogenates. Three mice were used for each of the shorter experimental periods and four or more for each of the longer ones. All tissue samples were counted in windowless gas-flow counters(4) with a counter efficiency of 65%. Standard corrections were applied for geometry, resolving time, and self-absorption before the average of the duplicate plates was taken. In view of the high activity of the injected drug, statistically significant counts could still be detected in some tissues as long as one week after injection. Since this represented only a relatively small amount of drug, only values greater than twice the standard deviation of the counting background were considered as significant. Thus counts equivalent to less than 0.0075 μg of drug or metabolite per gram of tissue are

* This work was done under a contract between the Atomic Energy Commission and the University of Chicago. It was also aided in part by a grant from Motorola Inc. and from the Dr. Wallace C. and Clara A. Abbott Memorial Research Fund of the University of Chicago.

†The C^{14} used for the synthesis of this compound was obtained on allocation from the Isotope Div., U.S. A.E.C.

TABLE I. Tissue Distribution of Isonicotinic Acid Hydrazide and Its Metabolites.

Tissue	.5 hr	1 hr	4 hr	8 hr	24 hr	2 day	4 day	7 day
Blood	5.7	4.1	.96	tr	tr	tr	tr	tr
Brain	4.4	3.2	2.1	.20	0	0	0	0
Lung	6.6	5.2	2.2	.32	.19	tr	tr	tr
Skin	5.4	3.6	2.6	.57	.41	.39	0–tr	tr–1.6
Muscle*	5	2.7	.66	0	0	0	0	0
Kidney	11.9	8	1.9	.21	tr	tr	tr	0–tr
Liver	10.6	8.9	4.1	2.3	.62	.42	.23	.14
Bile†	.09	.14	.19	.07	0	0	0	0
Feces	3.5	6.6	19.6	14	.25	0	0	0

* Adrenals, abdominal fat, heart, lymph nodes, sex organs and spleen distribution pattern similar to muscle.

† Bile reported as μg equivalents aspirated from gall bladder. All other data reported as μg equivalents/g of tissue or ml of blood. Each value represents avg of 3 or more mice, the variation in tissue concentration between mice was less than 10%.

considered as insignificant while values up to 0.015 μg are reported as trace quantities.

The total radioactivity measured in the tissues represents both that due to the drug and to its metabolites. However, since the metabolites have not all been characterized, the data are reported in terms of microgram equivalents of injected drug. A microgram equivalent is defined as the tissue radioactivity equivalent to one microgram of isonicotinic acid hydrazide.

In order to fractionate metabolites of the drug, chromatograms of tissues and excreta were run on Whatman No. 1 paper strips with a solvent system of butanol saturated with water. It was found that extraction of the urine with isobutyl alcohol yielded the same number of metabolites on the chromatograms as did direct application of the urine to the paper strips. Since they could not be applied directly, homogenates of tissues were filtered and extracted with isobutyl alcohol before being applied to the strips. The $C^{14}O_2$ was measured by means of an ionization chamber and vibrating reed electrometer(5). Current readings were converted to dps and then to cpm as measured by the windowless gas-flow counters.

Results. The data obtained in the distribution study are presented in Table I. Total recoveries from tissues and from excreta of injected activity ranged from 88-97%. Following subcutaneous injection, the drug is rapidly absorbed and distributed throughout the body but is retained in significant amounts by only a few tissues after 8 hours.

The activity in the blood was found both in plasma and in the saline washed cellular elements. Peak concentrations of activity were obtained in the blood shortly after subcutaneous injection of the labeled drug. After 8 hours only trace amounts of activity were detectable but these levels were maintained in the blood for as long as one week.

The lung contained more activity per gram of tissue than the blood per milliliter at each experimental period. Approximately 0.5% of the injected dose, or 1.1 μg equiv., was concentrated in the lung within the first hour. By 8 hours only 0.04% of the dose was present but trace amounts of activity were detectable for as long as one week.

During the first hour, the brain had less activity per gram of tissue than the blood per milliliter; however, it retained relatively more activity than the blood during the next 7 hours indicating a movement of drug or metabolite from blood into brain tissue. By 24 hours after injection, however, the brain no longer contained any detectable activity. Chromatograms of brain homogenates showed that both isonicotinic acid hydrazide and isonicotinic acid are present. Preliminary evidence obtained in these studies indicates that other metabolites containing the labeled carboxyl group do not enter the brain.

The liver took up large amounts of the drug and retained more on a per gram basis than any other organ throughout the experimental period. 9.8% of the injected dose was present in the liver after one-half hour while 1.8% was present by 8 hours. At the end of

a week 0.9% of the injected activity was still detectable in the liver.

The skin sample was taken from the back of the neck to eliminate the possibility of contamination from the injection site. Similar results to those reported were obtained with mice receiving intraperitoneal injections of the drug. The results obtained with skin samples were less consistent during the longer experimental periods than they were during the first 48 hours. Only one mouse in four showed more than trace activity in the skin at 4 days, while two out of four showed more than trace activity at one week after administration of the drug.

Shortly after injection such highly vascular organs as the spleen and the adrenal glands contained practically equal amounts of activity as the blood on a gram-ml basis; however, tissues such as striated muscle, heart, and abdominal fat were usually considerably lower. The activity detectable in the lymph nodes during the first few hours after injection was consistently lower than that of any other tissue. Muscle taken from near the injection site was equal in activity to other muscle tissue by 4 hours.

The data on the feces are reported on a per gram basis of the formed feces found in the lower bowel. The highest concentration of activity was noted during the 4 to 8 hour period. Direct plates of bile aspirated from the gall bladder show the highest activity during the 1- to 4-hour period. Excretion studies revealed that from 2-8% of the injected dose is excreted through the gastro-intestinal tract during the first 24 hours while from 1-3% is excreted during the following 48 hours.

The high activity detected in the kidneys during the first hour reflects the early excretion of the drug by this organ. Trace amounts of activity were detectable in the kidney throughout the experimental period. It was found that from 75-90% of the injected activity was excreted in the urine during the first 24 hours. Radioautographs made of chromatograms of the urine revealed the presence of unchanged drug plus 5 other metabolites. The distribution of activity on the chromatograms indicated that approximately 26% of the radioactivity in the urine results from unchanged drug while 55% is due to isonicotinic acid.

TABLE II. CPM per 100 ml Exhaled CO_2.

1–8 hr	8–16 hr	16–24 hr
25.6	17.9	14.3

When the exhaled CO_2 was precipitated as $BaCO_3$ and plated directly(6), no significant count could be detected with the windowless gas-flow counters. However, when larger volumes of CO_2 were counted in the ionization chamber, the count shown in Table II was obtained. If one considers the average CO_2 exhalation of a 20 g mouse as 0.05 mole per 24 hours(7), then less than 1% of the injected radioactivity was expired through the lungs during this period.

Discussion. The distribution and excretion studies indicate that labeled isonicotinic acid hydrazide is rapidly absorbed following subcutaneous injection, transported via the blood stream and excreted chiefly through the kidneys.

In view of the therapeutic use of isonicotinic acid hydrazide, it is highly significant that the lung always contains more activity per gram of tissue than any but the kidney, liver and skin. Since this drug has been found to be bacteriostatic *in vitro* against *M. tuberculosis* H37Rv at a concentration of 0.015 μg/ml(8), it is also noteworthy that the lung retains activity approximately equivalent to this amount of drug per gram of tissue for as long as a week after injection. The observation that the lung contains significant amounts of activity even with low blood levels during the period from 8 to 24 hours bears further investigation.

The apparent stimulation of the central nervous system by this drug(9) could be associated with the presence in the brain itself of the unchanged drug or its major metabolite. A more precise localization of the activity in the brain is now being studied both by dissection and by radioautograph of brain slices.

The localization of the drug in the skin presents a problem for further study in other species. In view of the relative size of the

skin as a body organ, the results given would suggest that the skin serves as a major storage depot for the drug. Whether the drug is retained here by combination with some fatty element or by the relatively high water content of the skin is at present unknown.

The gradual increase in the activity noted in the formed feces in the bowel, with the peak concentration at about four hours, results from both the increased output of activity through the bile during the previous two hours and the constipating effect of the drug. The mice generally held back both urine and feces during the first 4 hours following subcutaneous injection of the drug.

Summary. 1. Tissue distribution studies of labeled isonicotinic acid hydrazide in mice indicate that the drug is localized chiefly in the liver, skin, lung, brain and kidneys. 2. The lung contains significant amounts of activity even after the blood level has fallen to trace amounts. 3. Both isonicotinic acid hydrazide and its major metabolite isonicotinic acid are found in brain tissue. 4. The skin may serve as a major storage depot for the drug. 5. The urinary system is the major pathway for the excretion of the drug. 6. Less than 1% of the injected drug is converted to carbon dioxide.

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